A MUTATIONAL ANALYSIS OF THE ROLES OF CYTOPLASMIC DOMAINS OF THE GONADOTROPIN-RELEASING HORMONE RECEPTOR IN COUPLING AND INTERNALIZATION

by

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ABSTRACT

The G protein-coupled receptor (GPCR) family is the largest group of homologous proteins in the human genome. GPCRs are of prime physiological and medical importance as the actions of a wide range of hormones and drugs are mediated by these receptors. The gonadotropin-releasing hormone (GnRH) receptor is a member of the GPCR family, and plays a central role in the reproductive system. GnRH analogues are used therapeutically in a number of human disorders.

All GPCRs contain 7 largely α-helical transmembrane domains. An arginine residue located at the cytosolic boundary of the third transmembrane domain is conserved in all members of the rhodopsin-like subfamily of GPCRs, and is nearly always preceded by an acidic residue (DR motif). This arginine has been proposed to play a critical role in receptor activation. In this thesis, the effects of mutating these residues (Asp^{138} and Arg^{139} respectively, in the mouse GnRH receptor) to neutral amide residues, on coupling of the mouse GnRH receptor, were examined. In addition, the relationship of coupling to internalization in these mutant receptors was explored.

Mutation of Arg^{139} to Gln (R139Q) resulted in an 8-fold decrease in affinity for GnRH, and a similar decrease in affinity for a GnRH antagonist. Interestingly, the R139Q GnRH receptor was overexpressed 6- to 10-fold compared with wild type. In spite of this increased receptor number, the R139Q mutant showed a 150-fold decrease in potency of GnRH for stimulation of inositol phosphate production, as well as a decreased maximal response, indicating poor coupling to its effector system, with a calculated coupling efficiency of 6% relative to wild type. The modulation of agonist binding by temperature in this mutant receptor was investigated. Low temperature has been previously reported to induce a conformation with high agonist affinity in some GPCRs, resembling the activated state, and it was therefore of interest to determine whether this uncoupled mutant would display this transition. Despite its

severe uncoupling, agonist binding of the R139Q mutant showed modulation by temperature similar to the wild type receptor. Receptor-mediated internalization of GnRH agonist by the R139Q GnRH receptor was decreased to 38-52% of the wild type level and was not restored by protein kinase C activation with phorbol ester. These results indicate that the highly conserved Arg^{139} residue of the GnRH receptor plays critical roles in both G protein activation and receptor internalization, but not in temperature modulation of agonist binding.

The R139K, R139H and D138A GnRH receptor mutants showed markedly decreased agonist and antagonist binding, and agonist-stimulated inositol phosphate production, indicating either gross misfolding, and/or failure of expression of these mutant receptors. The D138N GnRH receptor showed normal agonist and antagonist binding affinities, and increased coupling efficiency and internalization, despite the evolutionary conservation of this acidic residue. The enhanced coupling efficiency supports the hypothesis that an ionic interaction between the Asp and Arg side chains of the DR motif holds the GPCR in the inactive conformation.

The DAB cross-linking method was used for the first time for measuring the internalization of agonist- and antagonist-occupied GPCRs. This method utilizes the enzymatic activity of horse-radish peroxidase to polymerize 3, 3'-Diaminobenzidene in endosomes, with the consequent cross-linking of endosomal content proteins (including radioligand-GnRH receptor complexes) to the DAB polymer. Using this method, antagonist-occupied GnRH receptors were shown not to undergo internalization, suggesting that receptor activation is a critical determinant for GnRH receptor internalization.

The chicken GnRH receptor is notable for having a cytoplasmic C-terminal tail, which is not present in the mammalian GnRH receptors. In order to investigate the role of the cytoplasmic tail of the chicken GnRH receptor in mediating its agonist-promoted internalization, a series of mutant constructs were prepared, in which

portions of the cytoplasmic tail were deleted, or Ser and Thr residues point mutated to Ala. Internalization of the receptor-ligand complex was then assessed by measuring the rate of internalization of bound radioligand. Truncations of the cytoplasmic tail resulted in receptors that internalized GnRH agonist at a lower rate than the wild type chicken receptor. Point mutations were made to further localize the tail elements critical for the internalization process. The data indicate that rapid agonist-promoted internalization of the chicken GnRH receptor is mediated through elements in the cytoplasmic C-terminal tail, distal to or including Ser^{337}. In addition, the results show that a Thr-Thr doublet located near the C-terminal end are the most important. Comparison of the data presented in this thesis with those obtained for other GPCRs shows that the motif Thr/Ser-Thr/Ser-Ile/Leu/Val may be an internalization sequence. Furthermore, these findings suggests that elimination of the cytoplasmic C-terminal tail during evolution of mammalian GnRH receptors may be related to its effects on internalization.

The effects of co-expressing dynamin and a dominant-negative mutant of dynamin on agonist promoted internalization of the chicken GnRH receptor were also investigated. The results suggest that internalization of the chicken receptor proceeds via a clathrin- and dynamin-dependent pathway, while human receptor internalization is dynamin-independent.

A simple mathematical model describing receptor trafficking was derived for the analysis of receptor internalization data. This model assumes that the rate of receptor degradation is small compared to the rates of receptor recycling and internalization, and can be neglected. On the basis of results obtained from photoaffinity-labeling studies of the mouse GnRH receptor, this assumption was justified. The covalent cross-link between the photoaffinity ligand ^{125}I-[*N*-azidobenzoyl-D-Lys^6]GnRH and the GnRH receptor was shown to be stable enough to allow the measurement of the rate of intracellular degradation of the mouse GnRH

receptor, which was shown to have a half-life of 4.8 hrs, corresponding to a degradation rate of 0.24%.min^{-1}.

DEDICATION

This thesis is dedicated to my parents, Jack and Marjorie Pawson, who have encouraged and supported me in all my endeavours.

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LIST OF PUBLICATIONS

Publications based on the work described in this thesis and on related topics are listed below.

1 Davidson, J.S., Assefa, D., **Pawson, A.J.**, Davies, P., Hapgood, J., Becker, I., Flanagan, C., Roeske, R and Millar, R.P. (1997) Irreversible activation of the gonadotropin-releasing hormone receptor by photoaffinity cross-linking: localization of attachment site to Cys residue in N-terminal segment. Biochemistry 36, 12881-12889.

2 **Pawson, A.J.**, Katz, A., Sun, Y.-M., Illing, N., Lopes, J., Millar, R.P. and Davidson, J.S. (1998) Contrasting internalization kinetics of human and chicken gonadotropin-releasing hormone receptors mediated by C-terminal tail. J. Endocrinol. 156(3), R9-R12.

3 Millar, R.P., Troskie, B., Sun, Y., Ott, T., Wakefield, I., Myburgh, D., **Pawson, A.J.**, Davidson, J.S., Flanagan, C., Katz, A., Hapgood, J., Illing, I., Weinstein, H., Sealfon, S.C., Peter, R.E., Terasawa, E, and King, J.A. (1997) Plasticity in the structural and functional evolution of GnRH: a peptide for all seasons. XIII International congress of comparative endocrinology, Yokohama, Japan, 15-27.

4 Myburgh, D.B., **Pawson, A.J.**, Davidson, J.S., Flanagan, C.A., Millar, R.P., and Hapgood, J.P. (1998) A single smino acid substitution in transmembrane helix VI results in overexpression of the human gonadotropin-releasing hormone receptor. Eur. J. Endocrinol., 139, 438-47.

5 Assefa, D., **Pawson, A.J.**, McArdle, C.A., Millar, R.P., Flanagan, C.A., Roeske, R. and Davidson, J.S. A gonadotropin-releasing hormone peptide antagonist with photoreactive group at position 1 cross-links to the N-terminal domain of the GnRH receptor: implications for antagonist binding. (Submitted).

6 Millar, R.P., Assefa, D., Ott, T., **Pawson, A.J.**, Troskie, B., Wakefield, I. and Katz, A. (1998) GnRH and GnRH analogues: Structure, actions and clinical applications. (Submitted).

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LIST OF ABBREVIATIONS USED

βARK	β-adrenergic receptor kinase
B_{max}	calculated receptor number
B_o	radioligand bound in the absence of competing unlabeled ligand
BSA	bovine serum albumin
CCV	clathrin coated vesicle
cDNA	complimentary deoxyribonucleic acid
DMEM	Dulbecco's modified Eagles medium
DTPA	diethylenetriaminepentaacetic acid
EC_{50}	peptide concentration required to half-maximally stimulate production of inositol phosphates
EDTA	ethylenedaminetetraacetic acid
FCS	fetal calf serum
G protein	guanine nucleotide binding protein
GnRH	gonadotropin-releasing hormone
GnRHR	gonadotropin-releasing hormone receptor
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	high performance liquid chromatography
IC_{50}	peptide concentration required to half-maximally inhibit binding of labeled GnRH peptides
IP	inositol phosphate
IP_{max}	maximal inositol phosphate response
IP_3	inositol 1,4,5-trisphosphate
K_d	dissociation constant
kDa	kiloDalton
mGnRH	mammalian GnRH
mRNA	messenger ribonucleic acid

PBS	phosphate-buffered saline
PKA	protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylmethylsulfonyl fluoride
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
WT	wild type

GnRH RECEPTOR NOMENCLATURE

The species of GnRH receptor is indicated by the first (lowercase) letter. m=mouse, h=human, and c=chicken. The mutation is indicated using the single-letter amino acid code.

Example: mD138A refers to mutation of Asp^{138} to Ala in the mouse GnRH receptor

GENERIC RESIDUE NUMBERING

The generic residue numbering system of Ballesteros and Weinstein (1996) is used in this thesis. In this system, the most conserved residue in a particular transmembrane domain is designated by the index 50. Thus the conserved Arg at the cytoplasmic side of helix 3 is numbered 3.50. The other residues are numbered in relation to the conserved residue. For example, the Asp directly adjacent to $Arg^{3.50}$ is denoted $Asp^{3.49}$, while the Ser is denoted $Ser^{3.51}$.

1 INTRODUCTION AND REVIEW OF LITERATURE

1.1 SUMMARY

G protein-coupled receptors (GPCRs) represent a large family of integral membrane proteins that transduce extracellular stimuli to the cells interior. Cellular responses to agonists of GPCRs become desensitized (attenuated) over time. Following agonist exposure, most GPCRs studied to date undergo agonist-promoted internalization. The mechanisms of this desensitization and agonist-promoted internalization are incompletely understood, and are the subject of this thesis.

In principle, desensitization could result from: (a) *uncoupling* of receptors from their cognate G proteins; (b) a decrease in the number of receptors on the cell surface as a result of their *internalization* (sequestration); (c) a decrease in the total cellular complement of receptors, which is referred to as *downregulation* and could result from decreased synthesis or increased degradation of receptors; (d) modulation of signaling downstream of the receptor (for example, concentration of G proteins or effector enzymes).

The first part of this introduction (**Section 1.2**) reviews desensitization of GPCRs, while the second part (**Section 1.3**) reviews what is known about internalization of GPCRs. It discusses agonist-promoted internalization and examines some of the proteins that are implicated in this process. The relationship between agonist-promoted internalization and G protein coupling is also explored. The final part of the introduction (**Section 1.4**) deals specifically with the GnRH receptor and reviews internalization, G protein coupling and desensitization in relation to its functioning.

1.2 DESENSITIZATION OF G PROTEIN-COUPLED RECEPTORS

1.2.1 Homologous and heterologous desensitization

Two types of desensitization may be distinguished, based on the mechanism by which they attenuate signaling of GPCRs. *Homologous desensitization* requires agonist-occupancy and activation of the desensitized receptor. G protein-coupled receptor kinase (GRK)-mediated phosphorylation and arrestin binding is one well-described mechanism for homologous desensitization (**Section 1.2.2.1**). *Heterologous desensitization* refers to the desensitization of receptors resulting from activation of *different* receptors, and does not require agonist occupancy of the desensitized receptor. Activation of second messenger-dependent kinases (PKA/PKC) is known to mediate heterologous desensitization of GPCRs (**Section 1.2.2.2**).

1.2.2 Molecular determinants of desensitization

1.2.2.1. The G protein-coupled receptor kinases (GRKs)

Six members of the GRK subfamily (Table 1) of Ser/Thr protein kinases have been identified and share 53-93% overall sequence homology (for review of functional and structural aspects see Sterne-Marr and Benovic, 1995). Unlike the second messenger-dependent kinases, there are no well-defined consensus sites for GRK-mediated phosphorylation of GPCRs. Studies of GPCR phosphorylation are generally complicated by the fact that in many cases both GRKs and second messenger-dependent protein kinases contribute to phosphorylation of the same receptor.

TABLE 1 CHARACTERISTICS OF GRKs

Family name	Common name	Known receptor substrates
GRK1	Rhodopsin kinase	rhodopsin, β_2-adrenergic
GRK2	βARK1	β_2-, α_{2A}-, α_{1B}-adrenergic, rhodopsin, m1 and m2 muscarinic, neurokinin-1, thrombin, dopamine, secretin, opioid, A2 and A3 adenosine, AT_{1A}
GRK3	βARK2	β_2-,α_{1B}-adrenergic, m2 muscarinic, neurokinin-1, thrombin, neurokinin-1, secretin
GRK4	IT-11	β_2-adrenergic, luteinizing hormone
GRK5		β_2-,α_{1B}-adrenergic, rhodopsin, secretin
GRK6		β_2-,α_{1B}-adrenergic, rhodopsin

Two phosphorylation domains, one for PKA in the third intracellular loop and one for GRK2/3 in the cytoplasmic tail, have been identified on the β_2-adrenergic receptor (Hausdorff et al., 1989). Ozcelebi et al. (1996) have demonstrated that both PKC and GRKs are capable of phosphorylating the cholesystokinin-A receptor, depending in which cell line it was expressed. Furthermore, both PKC and GRK2 have been shown to phosphorylate the m1 muscarinic receptor (Haga et al., 1996). Similar results were reported for the AT_{1A} receptor (Opperman et al., 1996).

Many GPCRs contain several potential GRK phosphorylation sites in their cytoplasmic tails, while others with shorter cytoplasmic tails contain few or no serine and threonine residues. The latter group of receptors may have long third intracellular loops containing multiple serine and threonine residues which are putative sites for GRK-mediated phosphorylation. Indeed, deletion of the Ser/Thr-rich region of the third intracellular loop of the α_{2A}-adrenergic receptor, almost completely abolished receptor phosphorylation and prevented short term agonist-promoted desensitization (Liggett et al., 1992). It is now known that the sites for

GRK-mediated phosphorylation of the α_{2A}-adrenergic receptor are four consecutive Ser residues in its third intracellular loop (Eason et al., 1995). Similarly, the sites required for GRK-mediated phosphorylation of the m2 muscarinic receptor have been identified as serine and threonine residues within its third intracellular loop (Nakata et al., 1994). It has also been demonstrated that GRK2/3 can phosphorylate the neurokinin-1 receptor (Kwatra et al., 1993). More recently it was reported that substance P induced homologous desensitization and internalization of the neurokinin-1 receptor in neurons, and that this may be mediated by GRK2 and GRK3 (McConalogue et al., 1998).

Through *in vitro* phosphorylation studies, tentative GRK2 and GRK5 phosphorylation sites have been identified in the β_2-adrenergic receptor (Fredericks et al., 1996). However, a recent report in which these Ser/Thr residues were mutated to Ala has demonstrated that neither desensitization nor the extent of receptor internalization was affected (Seibold et al., 1998), suggesting that sites other than those originally identified are involved.

Phosphorylation of Ser/Thr residues within the cytoplasmic C-terminal tail of a number of GPCRs has been implicated in their desensitization (Bouvier et al., 1988; Lattion et al., 1994; Hukovic et al., 1998). In contrast, for other GPCRs this region is required for their agonist-promoted internalization and not desensitization (Benya et al., 1993; Huang et al., 1995; Thomas et al., 1995b). Interestingly, Widmann et al., 1997 have reported that both homologous desensitization and agonist-promoted internalization of the glucagon-like peptide-1 receptor is dependent on phosphorylation at the same three serine doublets of the cytoplasmic C-terminal.

The requirement of cytoplasmic tail Ser/Thr residues for luteinizing hormone receptor desensitization was demonstrated by truncating the receptor at position 631. The resulting mutant was unable to desensitize (Sánchez-Yagüe et al., 1992). In contrast, a later study reported that when the cytoplasmic tail was truncated at

position 628, the truncated receptor desensitized to the same degree as the full-length wild type receptor (Zhu et al., 1993). The authors speculated that the reason for this discrepancy may be as a result of the newly created bulky arginyl-arginine carboxyl terminus of the 631stop truncation mutant, which could have conferred non-specific desensitization on this receptor. Apparently, positively charged polypeptides are able to non-specifically activate G proteins (Antonelli et al., 1991).

In several studies, the role of GRKs in mediating desensitization has been established through the co-expression of either the kinase or a dominant-negative mutant thereof with a particular receptor. Pippig et al. (1993) demonstrated that β_2-adrenergic receptor desensitization was enhanced by the overexpression of GRK2. Similar findings were reported, following the co-expression of a GRK, for the β_1-adrenergic (Freedman et al., 1995), α_{1B}-adrenergic (Diviani et al., 1996), α_2-adrenergic (Jewell-Motz and Liggett, 1996), thrombin (Ishii et al., 1994), AT_{1A} (Opperman et al., 1996), A3 adenosine (Palmer et al., 1995), m2 muscarinic (Schlador and Nathanson, 1997), and δ-opioid (Pei et al., 1995) receptors. Furthermore, in a recent study the effect of co-transfection of GRKs 1-5 on secretin receptor desensitization was evaluated (Schetzline et al., 1998). It was reported that GRKs 2 and 5 were the most potent at mediating secretin receptor desensitization, with GRK3 having a partial effect and GRKs 4 and 6 no effect.

Co-expression of a dominant-negative mutant of GRK2 was shown to prevent desensitization of the β_2-adrenergic (Kong et al., 1994), δ-opioid (Pei et al., 1995), and κ-opioid receptors (Raynor et al., 1994). Similar results were reported for β_1-adrenergic (Freedman et al., 1995), m2 muscarinic (Pals-Rylaarsdam et al., 1995), AT_{1A} (Opperman et al., 1996), and A2 adenosine (Mundell et al., 1997) receptors, following co-expression of GRKs.

It has been observed that differential receptor trafficking and desensitization properties exist for the μ-opioid receptor, depending on the agonist activating the receptor (Zhang et al., 1998). It is suggested that these properties are related to the differential abilities of the various agonists to induce GRK-mediated μ-opioid receptor phosphorylation. Under normal expression conditions, both etorphine and morphine activated the receptor, but only etorphine stimulated its phosphorylation and consequent arrestin- and dynamin-dependent internalization (Zhang et al., 1998). A recent study demonstrated that when GRK2 was overexpressed, morphine was also able to induce μ-opioid receptor phosphorylation, arrestin binding, desensitization, and agonist-promoted internalization (Zhang et al., 1998).

The identity of the kinase(s) responsible for mediating m3 muscarinic receptor phosphorylation have eluded investigators for some time (Tobin et al., 1992; Tobin and Nahorski, 1993). Recently, it was demonstrated that m3 muscarinic receptors expressed in insect cells were phosphorylated by GRK2 in an agonist-dependent manner. This was the first demonstration that m3 muscarinic receptors could serve as substrates for GRKs (Debburman et al., 1995). A novel class of receptor kinase (designated MRK for muscarinic receptor kinase) that mediates agonist-dependent phosphorylation of the m3 muscarinic receptor has now been identified (Tobin et al., 1996).

1.2.2.2 **The second messenger-dependent protein kinases (PKA/PKC)**

The second messenger-dependent protein kinases participate in GPCR heterologous desensitization. GPCRs may contain phosphorylation sites for PKA, PKC, or calcium/calmodulin-dependent protein kinase, and some of these sites may lead to desensitization when phosphorylated (Lefkowitz, Stadel, and Caron, 1983; Lefkowitz and Caron, 1986, 1987; Sibley et al., 1986, 1987, 1988; Caron and Lefkowitz, 1993; Lefkowitz, 1993). In principle, it is possible that GPCRs are

phosphorylated by kinases of their own signaling cascade, or by kinases activated by other receptors. Thus heterologous desensitization is a potential means of "cross-talk" between signaling pathways. Some of the possible modulatory loops are illustrated in Figure 1.

The major mechanism of heterologous desensitization of adenylyl cyclase-coupled receptors is via phosphorylation by PKA. Early studies on GPCR desensitization demonstrated that PKA mediates the phosphorylation and heterologous desensitization of the β_2-adrenergic receptor (Stadel et al., 1983; Sibley et al., 1986, 1987). Therefore, any hormone or drug that leads to an increase in cAMP levels and adenylyl cyclase activity, will result in the activation of PKA and subsequent phosphorylation and desensitization of the β_2-adrenergic receptor. Thus this type of regulation of GPCRs is defined as heterologous.

PKC can also phosphorylate the β_2-adrenergic receptor as demonstrated by pre-treatment of intact cells with phorbol esters which directly activate PKC, leading to receptor phosphorylation and desensitization (Sibley et al., 1984). These results suggest that PKC, which is activated by phospholipase C-mediated hydrolysis of phospholipids promoted by a number of receptors, can also contribute to the phosphorylation and heterologous desensitization of the β_2-adrenergic receptor.

Phosphorylation and desensitization of the phospholipase C-coupled α_1-adrenergic receptor was demonstrated following phorbol ester activation of PKC (Leeb-Lundberg et al., 1985). Bouvier et al. (1986) have demonstrated through peptide mapping studies that both PKC and PKA are capable of phosphorylating the α_1-adrenergic receptor. A recent study testing whether PKC phosphorylation is a mechanism by which the α_{2A}-adrenergic receptor can be regulated by other receptors, reported that the selective activation of α_{1B}-adrenergic receptor co-expressed with the α_{2A}-adrenergic receptor, led to desensitization of the latter (Liang

1.2.2.3 **The arrestins**

The arrestin family has six members, including the visual and non-visual isoforms (Palczewski, 1994; Krupnick and Benovic, 1998). The arrestins bind preferentially to GRK-phosphorylated GPCRs (as opposed to second messenger- or non-phosphorylated receptors) and mediate homologous or agonist-dependent desensitization by disrupting the interaction between activated receptor and cognate G protein (for a study of the kinetics of arrestin interactions with GPCRs see Gurevich et al., 1995).

Mechanisms have been elucidated by which GRKs are specifically targeted to the membrane-bound receptors. These involve their modification by isoprenylation or palmitoylation and interaction with G protein $\beta\gamma$ subunits (Pitcher et al., 1992). In contrast, the mechanism(s) by which arrestins are targeted to the plasma membrane to bind and uncouple GPCRs remains unknown. Furthermore, the regions of GPCRs required for arrestin interaction have not yet been delineated. Ferguson et al. (1996a) have suggested that neither the cytoplasmic tail nor GRK-mediated phosphorylation is an absolute requirement for interaction of either β-arrestin-1 or β-arrestin-2 with the β_2-adrenergic receptor.

1.2.2.4 **The MAP kinase pathway**

A recent study has reported that, in HEK293 cells expressing dominant-negative mutants of β-arrestin or dynamin, β_2-adrenergic receptor-mediated activation of MAP kinase was completely blocked (Daaka et al., 1998). This suggests that GRKs and β-arrestins, which uncouple GPCRs and target them for agonist-promoted internalization as discussed below, are essential in the GPCR-mediated MAP kinase signaling cascade. It has also been suggested that some feedback contribution from

the MAP kinase cascade is required for μ-opioid receptor desensitization (Polakiewicz et al., 1998).

1.3 INTERNALIZATION OF G PROTEIN-COUPLED RECEPTORS

Although receptor-mediated endocytosis is commonly thought of as a mechanism for the uptake of nutrients (for example, low density lipoprotein and transferrin) bound to specific receptors, it is also important in regulating the protein composition of the plasma membrane, and as discussed above, plays a role in attenuating the responses of signal-transducing receptors by removing the ligand-receptor complexes from the cell surface.

Cell surface receptors may be categorized into two types based upon their patterns of internalization. Receptors for nutrients such as those for low density lipoprotein and transferrin internalize and recycle back to the surface at essentially the same rate whether or not they are occupied by ligand (Trowbridge et al., 1993). They are constitutively clustered in coated pits and undergo rapid internalization in the presence or absence of ligand (Goldstein et al., 1985). In contrast, receptors such as the tyrosine-kinase receptors (for example, the epidermal growth factor receptor) and GPCRs are only concentrated in coated pits and internalized *after* binding ligand (Trowbridge, 1991).

In GPCRs, several studies have reported that antagonist ligands are internalized slowly or not at all, compared with agonists, suggesting a requirement for receptor activation in this process (Hoelscher et al., 1991; Lutz et al., 1992; von Zastrow et al., 1994; Williams et al., 1998). No internalization of V1 and V2 vasopressin (Lutz et al., 1992), and β_2-adrenergic receptors (von Zastrow et al., 1994) was detectable following the binding of antagonists. Deglycosylated human choriogonadotropin, which retained its ability to bind to the lutropin/choriogonadoropin receptor but was unable to stimulate the production of cAMP, was internalized at a much slower rate

than normal human choriogonadotropin (Hoelscher et al., 1991). Furthermore, antagonist-occupied gastrin-releasing hormone receptors did not undergo internalization, and treatment with PMA did not induce their internalization (Williams et al., 1998). These findings suggested that simple ligand binding is insufficient to initiate internalization of the ligand-receptor complex, and that agonist-induced receptor activation is a major determinant of the rate of internalization. Theoretical possible relationships between agonist occupancy and internalization are presented in Figure 2

In contrast, Roettger et al. (1997) have provided evidence that the cholecystokinin receptor undergoes internalization in response to antagonist occupation. Furthermore, while receptor phosphorylation has been suggested as a trigger for receptor internalization (see below **Section 1.3.2.2**), no significant increase in the phosphorylation state of the cholecystokinin receptor was reported following antagonist exposure. Thus, antagonist-stimulated internalization of the cholecystokinin receptor appears to proceed independently of receptor phosphorylation. Rather, it was suggested that antagonist occupation leads to a conformational change or stabilizes a conformation of the receptor that exposes a domain which mediates its internalization (Roettger et al., 1997). Two antagonists of the V2 vasopressin receptor exerted different effects on receptor internalization, as determined by confocal fluorescence microscopy. In this study (Pfeiffer et al., 1998), the nonpeptidic antagonist did not induce any visible receptor internalization, whereas the peptidic antagonist induced a slow but substantial receptor internalization (Pfeiffer et al., 1998).

Some uncoupled mutant receptors have displayed defective internalization (Strader et al., 1987; Cheung et al., 1989, 1990; Campbell et al., 1991; Lameh et al., 1992; Nussenzveig et al., 1993a; Hunyady et al., 1994c; Benya et al., 1994; Arora et al., 1995, 1996, 1997; Dhanwada et al., 1996, Laporte et al., 1996), although this relationship has not held true in all cases (Hausdorff et al., 1990; Cheung et al.,

1990; Lameh et al., 1992; Benya et al., 1993; Nussenzveig., 1993b). These results suggest that for some receptors, G protein coupling is necessary for efficient agonist-promoted internalization, and raises the question whether agonist-promoted internalization might be mediated by the activated effector system in these receptors.

The role of the activated effector system in mediating agonist-promoted internalization remains largely unexplored, especially in the case of uncoupled receptor mutants. Support for the proposal that the activated effector system (Figure 2) contributes to agonist-promoted internalization has come from studies showing that inhibition of G protein-phospholipase C interaction by the amino steroid U-73122 results in decreased internalization of thyrotropin-releasing hormone receptor (Nussenzveig et al., 1993b), and muscarinic receptors (Thompson et al., 1991).

1.3.1 **Pathways of agonist-promoted internalization**

There are now 5 known independent pathways for endocytic internalization: the clathrin-dependent pathway, the caveolar pathway, a clathrin- and caveolar-independent pathway, macropinocytosis, and phagocytosis (Reizman et al., 1998). So far, the only common feature of these different pathways is the requirement for an intact actin cytoskeleton. The predominant pathway for the agonist-promoted internalization of GPCRs is via the clathrin-dependent pathway. Internalization into clathrin-coated vesicles (CCVs) has been described for the β_2-adrenergic (Von Zastrow and Kobilka, 1992), thrombin (Hoxie et al., 1993) , neurokinin-1 and gastrin-releasing peptide (Grady et al., 1995a, b), and m1 muscarinic (Tolbert and Lameh, 1996) receptors.

Some reports have described a caveolin-dependent (or clathrin-independent) internalization mechanism for the β_2-adrenergic and cholesystokinin-A receptors (Dupree et al., 1993; Roettger et al., 1995). Two distinct mechanisms for agonist-

promoted GPCR internalization were reported in a study of β_2-adrenergic and AT_{1A} receptor internalization (Zhang et al., 1996a). This study reported that when clathrin-coated pit formation was blocked by the overexpression of a dominant-negative dynamin mutant (see **Section 1.2.2.3**), it did not prevent the internalization of the AT_{1A} receptor, while β_2-adrenergic receptor internalization was markedly reduced. This strongly suggests that certain GPCRs have the ability to utilize an alternative (clathrin-independent) internalization pathway. The mechanism(s) of the clathrin-independent pathway of internalization are unknown, but the existence of such a pathway, at least after inactivation of the clathrin pathway, is no longer in doubt.

The term **caveolae** is used to describe flask-shaped plasma membrane pits that are independent of clathrin coats (Peters et al., 1985). Caveolae, thought to be formed by the accumulation of the 22 kDa integral membrane protein caveolin, have been associated with the endocytic transport of solutes across the cell, rather than transport to endosomes, an activity usually associated with CCVs (Milici et al., 1987). Although caveolin is proposed to be a component of the caveolar "coat", a lack of caveolin expression has not been shown to cause a lack of caveolae. Indeed, one study suggests that caveolin may be a regulatory component of caveolae, rather than necessary to maintain their structure (Fra et al., 1994). A report demonstrating an interaction between caveolin and G proteins (Li et al., 1995), suggests that caveolin could negatively regulate the activation state of G proteins, or put another way, stabilize G proteins in an inactive state.

Therefore, it appears that in many cases receptor internalization can be accomplished by more than one pathway, but the preferred mechanism of internalization utilized by a particular GPCR is probably dependent upon both receptor-specific structural determinants and the cellular environment in which it is expressed (Von Zastrow and Kobilka, 1992). The functional implications if any, of these independent mechanisms for agonist-promoted internalization are not known.

1.3.2 **Molecular determinants of agonist-promoted internalization**

1.3.2.1 **Clathrin, the AP-2 complex and its components**

The basic structures of the coats of CCVs are formed by hexagonal and pentagonal lattices made up by the functional cytosolic unit, the clathrin triskelion (Pearse and Robinson, 1990) (Figure 3). This unit consists of three heavy chains (180 kDa each) complexed with three light chains (30-35 kDa each). The heavy chains comprise two distinct domains, an amino-terminal globular domain, generally referred to as the terminal domain, which lies at the distal end of each triskelion leg (Figure 3). In the coated pit, the terminal domains are believed to be located beneath each triskelion vertex (centre) of the lattice above and to project toward the plasma membrane (Krupnick et al., 1997a, b) (Figure 3). The remaining domain forms the triskelion core, consisting of the vertex, the proximal leg, and a portion of the distal leg. One clathrin light chain is associated with each proximal leg (Pearse and Robinson, 1990).

CCVs also contain non-clathrin components, the adaptors. The two adaptor complexes that have so far been identified, are the AP-1 and AP-2 complexes (a relative of AP-1 and AP-2 has been recently discovered and termed AP-3, but this complex is thought to function independently of clathrin (Mellman, 1996). These complexes play a critical role in the attachment of clathrin to membranes (Mellman, 1996). The AP-1 adaptor is a component of the *trans*-Golgi network clathrin coats and consist of four subunits: γ- and β1-adaptins, μ1 and σ1. The AP-2 complex, found at the plasma membrane where it is associated with clathrin coats, also comprises four related subunits: α- and β2-adaptins, μ2, and σ2 (Pearse and Robinson, 1990; Mellman, 1996). It has been proposed that upon binding, the adaptor complexes could induce the membrane curvature that optimally facilitates

the clathrin-mediated formation of coated pits (Mellman, 1996). Apart from their role in linking the clathrin lattices to the plasma membrane, the α- and β2-adaptins have also been implicated in the recognition of sorting signals in the cytoplasmic tails of many integral membrane proteins (Pearse, 1988; Glickman et al., 1989; reviewed in Mellman, 1996). More recent evidence suggests that μ2 of the AP-2 complex may be directly involved in sorting signal recognition (Ohno et al., 1995). Furthermore, a recent study has shown that the envelope glycoprotein (Env) of human immunodeficiency virus, type 1 (HIV-1), which undergoes rapid internalization, binds specifically to μ2, as well as the complete AP-2 complex, via its cytoplasmic domain (Boge et al., 1998).

1.3.2.2 **The serine/threonine kinases and β-Arrestins**

The idea that receptor phosphorylation might not only be required to uncouple GPCRs (see **Section 1.2**), but also play a critical role initiating receptor internalization, was first suggested by Sibley et al. (1986).

There are conflicting reports concerning the relationship between GPCR phosphorylation and consequent receptor internalization. Some studies (Table 2A) have shown that ligand-induced phosphorylation of serine/threonine residues is needed for efficient internalization of receptor-bound hormone (Lameh et al., 1992; Benya et al., 1993; Moro et al., 1993; Wang et al. 1997, Widmann et al., 1997).

Other studies (Table 2B) have shown that phosphorylation is **not** involved in internalization (Cheung et al., 1989; Hunyady et al., 1994a; Holtmann et al., 1996), and indeed early reports argued against a role for phosphorylation in the internalization of the β_2-adrenergic receptors (Bouvier et al., 1988; Hausdorff et al., 1989; Campbell et al., 1991; Kong et al., 1994).

TABLE 2 STUDIES ADDRESSING THE RELATIONSHIP OF GPCR PHOSPHORYLATION TO INTERNALIZATION

A: Studies showing phosphorylation is necessary for internalization

Receptor	Reference
β_2-adrenergic	Ferguson et al., 1995, 1996a; Ménard et al., 1996; Ruiz-Gómez and Mayor, 1997
bombesin	Tseng et al., 1995
D_2-dopamine	Itokawa et al., 1996
gastrin-releasing peptide	Benya et al., 1993
glugagon-like peptide-1	Widmann et al., 1997
interleukin-8	Richardson et al., 1998
lutropin/choriogonadotropin	Wang et al., 1997
m1 muscarinic	Lameh et al., 1992; Moro et al., 1993
m2 muscarinic	Tsuga et al., 1994; Schlador and Nathanson, 1997; Pals-Rylaarsdam et al., 1997
neurotensin	Hermans et al., 1996
parathyroid hormone	Huang et al., 1995

B: Studies showing phosphorylation is not necessary for internalization

Receptor	Reference
AT_{1A}	Hunyady et al., 1994a
β_2-adrenergic	Bouvier et al., 1988; Cheung et al., 1989; Hausdorff et al., 1989; Campbell et al., 1991; Kong et al., 1994
secretin	Holtmann et al., 1996

However, more recent studies of β_2-adrenergic (Ferguson et al. 1995, 1996a; Ruiz-Gómez and Mayor, 1997) and m2 muscarinic (Tsuga et al. 1994; Schlador and Nathanson, 1997) receptors indicated that agonist-induced phosphorylation facilitates the internalization of these two receptors. The latter study reported that GRK2 overexpression enhanced m2 muscarinic receptor internalization, and

overexpression of a dominant-negative GRK2 mutant (the K220W mutant) markedly retarded its internalization (Tsuga et al., 1994). However, it now appears that the results of this study were dependent on the cell-type being used, since inhibition of internalization was seen when COS-7 cells were used, but not in BHK-21 and HEK293 cells (Pals-Rylaarsdam et al., 1997).

A clear role for GRK2-mediated phosphorylation was demonstrated with the use of an internalization-defective β_2-adrenergic receptor mutant (the Y326A mutant) (Ferguson et al., 1995, 1996a). Even though this mutant receptor did not serve as an efficient substrate for GRK2, when GRK2 was overexpressed, it completely restored the mutant receptor's phosphorylation and internalization defects. However, GRK2 overexpression could not restore internalization when the putative GRK2 phosphorylation sites were mutated in the Y326A mutant receptor (Ferguson et al., 1995, 1996a). It has also been shown that the internalization of the β_2-adrenergic Y326A receptor mutant can be restored by overexpression of any GRK that has the ability to phosphorylate this mutant (Ménard et al., 1996).

A role for GRK-mediated phosphorylation was also demonstrated in D_2-dopamine receptor internalization (Itokawa et al., 1996). Furthermore, phosphorylation at Ser/Thr residues may mediate the internalization of the gastrin-releasing peptide, parathyroid hormone and parathyroid hormone-related protein, bombesin, and neurotensin receptors, since removal of their Ser/Thr-rich C-terminal cytoplasmic tails impairs their internalization (Benya et al., 1993; Huang et al., 1995; Tseng et al., 1995; Hermans et al., 1996). In addition, Richardson et al. (1998) have shown that a phosphorylation-deficient mutant of the interleukin-8 (IL-8) receptor A was resistant to IL-8-induced receptor internalization.

A mechanism by which phosphorylation would facilitate internalization of GPCRs has been suggested (Ferguson et al. 1995, 1996a). β-arrestins, which bind to GPCRs in response to GRK-mediated phosphorylation, are proposed to act as adapter-like

molecules serving to couple the receptor to the cells endocytotic machinery (Ferguson et al. 1996a, Zhang et al. 1996). Ferguson et al. (1996a) reported that overexpression of either β-arrestin-1 or β-arrestin-2 in HEK293 cells is sufficient to restore the ability of the β_2-adrenergic Y326A receptor mutant to internalize. In addition, arrestin overexpression was able to restore the defective internalization of a β_2-adrenergic receptor mutant in which the cytoplasmic tail was truncated at Cys^{341} to remove putative GRK phosphorylation sites (Ferguson et al., 1996a). The same study provided further evidence of a role for GRK-mediated receptor phosphorylation in promoting interactions with arrestins, since co-expression of small amounts of GRK2 with arrestins enhanced the arrestin-dependent recovery of Y326A internalization (Ferguson et al., 1996a).

Overexpression of GRK2 and β-arrestin-1 have been shown to synergistically promote m2 muscarinic receptor desensitization and internalization in JPEG-3 cells (Schlador and Nathanson, 1997). Furthermore, whereas the m2 muscarinic receptor was shown to undergo increased internalization when β-arrestin-1 and β-arrestin-2 were overexpressed (Pals-Rylaarsdam et al., 1997), the internalization of m1, m3, and m4 muscarinic receptors was unchanged, even when a dominant-negative mutant of arrestin was used to interrupt endogenous arrestin function (Lee et al., 1998), suggesting that m1, m3, and m4 muscarinic receptors utilize arrestin-independent internalization pathways. In a recent study it was demonstrated that substance P, an agonist of the neurokinin receptor, induced internalization of neurokinin-1 receptor in neurons, and that this may be mediated by GRK2 and GRK3 in addition to β-arrestins-1/2 (McConalogue et al., 1998).

Pitcher et al. (1998) have demonstrated that GRK2 is a microtubule-associated protein, and report that it specifically phosphorylates tubulin *in vitro*. These results suggest a new role for GRK2; as an effector of GPCR activation on cytoskeleton

organization. The implications of this finding on the internalization process of GPCRs are not yet clear.

The ability of arrestins to interact with clathrin has been demonstrated (Goodman et al., 1997; Krupnick et al., 1997a, b). These studies show that arrestin binds via its C-terminus to the terminal domain of clathrin, and is colocalized with the β_2-adrenergic receptor during the early stages of endocytosis (Goodman et al., 1996). Since the terminal domain is oriented toward the plasma membrane in coated pits, and binds arrestins and AP-2 complexes, it appears that arrestin acts as the anchor responsible for GPCR recruitment to the coated pit.

Recently, more information on the regulation of arrestin itself was reported (Lin et al., 1997). This study demonstrated that β-arrestin-1 is constitutively phosphorylated at a C-terminal Ser residue, after which it is recruited to the plasma membrane as a result of binding to agonist-occupied receptors. Once at the plasma membrane it becomes dephosphorylated in order to allow its interaction with clathrin and AP-2. Following receptor internalization, β-arrestin-1 is rephosphorylated. From these observations it appears that arrestin function is regulated by its phosphorylation state.

In summary, the main role of GRK-mediated phosphorylation in GPCR internalization seems to be to promote interactions with arrestins. Arrestins serve a dual regulatory role in the life cycle of GPCRs. They mediate rapid uncoupling by binding to GRK-phosphorylated receptors, and they target the receptors for internalization via the clathrin-coated pit endosomal pathway where receptor dephosphorylation and resensitization can occur (discussed in **Section 1.3.4.2**).

1.3.2.3 **Dynamin**

Dynamin, a 100 kDa GTPase, is now known to be a major component in clathrin-mediated internalization and has been shown to bind to α-adaptin of the AP-2 complex (Wang et al., 1995). Dynamin catalyzes the GTP-dependent disconnection of CCVs from the plasma membrane, by self-assembling into collar-like quarternary structures around the neck of the internalizing coated pit. It has been suggested that a concerted conformational change in these collars would bring about the disconnection of the budding vesicle from the plasma membrane (Hinshaw et al., 1995; Takei et al., 1995).

The importance of dynamin for the internalization of β_2-adrenergic receptors, has been demonstrated by the overexpression of a dominant-negative mutant of dynamin (the K44A mutant) which inhibited β_2-adrenergic receptor internalization (Zhang et al., 1996a). In contrast, in the same study AT_{1A} receptor internalization did not require dynamin. These results show that while β_2-adrenergic receptors internalize via the clathrin-dependent pathway as discussed above, the AT_{1A} receptor may be able to activate an alternative internalization pathway that is independent of both dynamin and β-arrestin (Zhang et al., 1996a). To investigate whether muscarinic receptors internalize in a subtype-specific manner via the dynamin-dependent CCV pathway, the effect of overexpressing the dominant-negative K44A dynamin mutant on the internalization of m1, m2, m3, and m4 muscarinic receptors was tested (Vögler et al., 1998). It was shown that while m1, m3, and m4 muscarinic receptor internalization was blocked by dominant-negative dynamin, that of the m2 subtype was unaffected, suggesting that different internalization pathways can be utilized by GPCRs, even within a closely related receptor group. These results also suggest that internalization of m1, m3, and m4 muscarinic receptors occurs via a dynamin-dependent, but arrestin-independent pathway (see **Section 1.3.2.2** above, Lee et al., 1998).

It has been shown that GTP-bound dynamin binds with high affinity to dissociated G protein βγ-subunits in neuroendocrine GH4C1 cells (Liu et al., 1997). This interaction inhibits the GTPase activity of dynamin, and may favour endocytic vesicle formation (Liu et al., 1997). Relevant to this, Takei et al. (1995) showed that the stabilization of dynamin in its GTP-bound state by GTPγS resulted in a lengthening of the vesicle neck to form long tubules as a result of progressive polymerization of dynamin.

A further study on domain structure and intramolecular regulation of dynamin has shown that dynamin is a tetramer, that the C-terminal Pro- and Arg-rich domain and the pleckstrin homology (PH) domain are, respectively, the positive and negative regulators of dynamin self-assembly and GTPase activity. The α-helical region between these two domains, now called the GTPase effector domain (GED), interacts with the N-terminal GTPase domain to stimulate GTP hydrolysis (Muhlberg et al., 1997).

1.3.2.4 **The Rab proteins**

The Rab family of Ras-related GTPases play important roles in membrane docking and fusion (Hall, 1990). Two members of this family have been implicated in the regulation of constitutively internalizing receptors. Overexpression of Rab5 increases the rate of transferrin receptor internalization (Bucci et al., 1992), while an overexpression of Rab4 was shown to accelerate the rate of recycling of the transferrin receptor back to the cell surface (van der Sluijs et al., 1992). Rab5 is therefore rate-limiting for the constitutive internalization of transferrin receptors. It is the best-characterized of the Rab GTPases and is required for the fusion between coated vesicles and early endosomes (Bucci et al., 1992). Very little is known about the relationship between Rab proteins and the agonist-promoted internalization of

GPCRs. Moore et al. (1995) demonstrated that the agonist-promoted internalization of β_2-adrenergic receptors proceeds in a clathrin-mediated and Rab5-dependent manner. These results suggest that the internalization pathways of constitutively internalizing receptors (for example, transferrin receptors) are also utilized by GPCRs, and it is possible that Rab5 may also be rate-limiting in the agonist-promoted internalization of GPCRs.

1.3.2.5 **The Rho and Rac proteins**

The three mammalian Rho proteins (A, B, and C) and the two Rac proteins (1 and 2) are Ras-related GTP-binding proteins and are known to be involved in the organization and assembly of the actin cytoskeleton (Madaule and Axel, 1985; Hall, 1993). Lamaze et al. (1996) have demonstrated roles for both Rho and Rac in the regulation of CCV formation and receptor-mediated endocytosis of the transferrin receptor through coated pits. A role for Rho but not Rac proteins in the control of constitutive fluid-phase endocytosis has been shown in *Xenopus* oocytes (Schmalzing et al., 1995). While there is as yet no evidence for a direct role of Rho and Rac in agonist-promoted internalization of GPCRs, their role in mediating endocytosis via clathrin-coated pits suggests their possible involvement.

1.3.2.6 **Ubiquitin**

It is well known that the modification of cytoplasmic proteins with polyubiquitin chains targets them for recognition and degradation in the multisubunit multicatalytic proteolytic complex known as the 26S proteosome (Goldberg and Rock, 1992; Ciechanover, 1994). The ubiquitination of cell surface receptors was first reported for the growth hormone receptor (Leung et al., 1987), and there is now also evidence that the ubiquitination of the growth hormone receptor and its internalization are closely related events (Strous et al., 1996).

It has been shown that a GPCR expressed in *S. cerevisiae*, the α-factor receptor, undergoes agonist-promoted ubiquitin-dependent internalization (Hicke and Reizman, 1996). As with many GPCRs, the α-factor receptor is constitutively internalized at a very slow rate in the absence of ligand. The binding of the peptide pheromone, α-factor, stimulates the signal transduction pathway, resulting in agonist-promoted internalization of the α-factor receptor. Reneke et al. (1988) and Hicke and Reizman (1996) have shown that in response to α-factor binding, the α-factor receptor becomes phosphorylated at cytoplasmic C-terminal tail Ser/Thr residues. Furthermore, the receptor becomes ubiquitinated at Lys residues within the cytoplasmic C-terminal tail. It was demonstrated that radiolabeled α-factor was not internalized in yeast mutants lacking the multiple enzymes required for ubiqitination, indicating that ubiquitination of the α-factor receptor is required for its internalization. Following the identification and mutational analysis of a nine amino acid internalization signal sequence (SINNDAKSS) in the cytoplasmic tail of the α-factor receptor, it was suggested that the serine residues within this sequence are critical sites for receptor phosphorylation, which in turn positively regulates receptor ubiquitination (Hicke and Reizman, 1996; Hicke et al., 1998). It is presently not clear how widely the ubiquitin internalization signal is utilized within the GPCR superfamily of receptors.

1.3.3 **Domains of GPCRs involved in agonist-promoted internalization**

1.3.3.1 **The DRYXXV/IXXPL sequence of the second intracellular loop**

The rhodopsin-like GPCRs exhibit conservation of certain residues in their transmembrane domains, which allows alignment of receptors within this family (Probst et al., 1992). An Arg residue, designated $Arg^{3.50}$ according to the generic residue numbering system of Ballesteros and Weinstein (1996), located at the interface between the third transmembrane domain and the cytoplasm, is conserved

in all members of the family, and is nearly always preceded by an acidic residue (Asp or Glu), and frequently followed by a Tyr (DRY motif). Because of its high degree of conservation, and its location at the membrane-cytoplasm interface, the Arg residue within this motif has been proposed to play a critical role in receptor activation (Oliveira et al., 1994; Scheer et al., 1996).

Evidence in support of a central coupling role for $Arg^{3.50}$ has come from site-directed mutagenesis of this residue, substitution of which has been consistently shown to result in uncoupling in several GPCRs (Franke et al., 1990, 1992; Ohyama et al., 1992; Min et al., 1993; Rosenthal et al., 1993; Benya et al., 1994; Zhu et al., 1994; Jones et al., 1995; Dhanwada et al., 1996). In contrast, mutation of the preceding acidic residue $Asp^{3.49}$/$Glu^{3.49}$ has produced a spectrum of results, ranging from complete abolition of coupling (Fraser et al., 1988) to constitutive activation of the effector system (Sakmar et al., 1989; Scheer et al., 1996).

A recent computational modelling study by Ballesteros and co-workers (Ballesteros et al., 1998) suggests that the orientation of the $Arg^{3.50}$ side-chain is modulated by conserved residues within TM3 surrounding $Arg^{3.50}$. In particular, it is proposed that the inactive receptor conformation is stabilized by an ionic interaction between $Glu/Asp^{3.49}$ and $Arg^{3.50}$, and that the $Glu/Asp^{3.49}$ becomes protonated upon receptor activation allowing the $Arg^{3.50}$ side-chain to be released and to participate in G protein activation. The strong conservation of the acidic residue at position 3.49 in GPCRs could imply that its protonation forms part of a general mechanism for receptor activation. The orientation of $Arg^{3.50}$ that is required for efficient receptor-G protein interaction is supported by a highly conserved β-branched Ile residue ($Ile^{3.54}$) in the α-helical turn following $Arg^{3.50}$. This model suggests that the conserved residues surrounding $Arg^{3.50}$ act like a cage that surrounds the Arg and affects its orientation, and predicts that disruption of the Arg-cage will impair receptor function (Ballesteros et al., 1998).

1.3.3.2 **The serine/threonine-rich third intracellular loop**

A Ser/Thr-rich region in the middle of the third intracellular loop of the m1 muscarinic receptor has been shown to be critical for its efficient agonist-promoted internalization (Maeda et al., 1990; Arden et al., 1992; Lameh et al., 1992; Moro et al., 1993). In addition, when residues 264-394 of the third intracellular loop of the m4 muscarinic receptor are deleted or a membrane proximal Thr (Thr^{399}) mutated to Ala, a markedly impaired internalization rate is observed (Van Koppen et al., 1994, 1995). The above studies all involved the removal or disruption of PKA phosphorylation sites. These observations provide evidence that receptor phosphorylation may play a role in muscarinic receptor internalization.

In contrast, in the case of the β_2-adrenergic receptor, the third intracellular loop does not seem to be critical for internalization, but rather for receptor-G protein coupling (Cheung et al., 1990; Hausdorff et al., 1990). Similarly, Nakamura et al. (1998) has recently shown that agonist-induced phosphorylation at Ser/Thr residues in the third intracellular loop of the rat follitropin receptor facilitates its uncoupling, but phosphorylation of these residues is not necessary for its agonist-promoted internalization. This study also demonstrated that the first intracellular loop is phosphorylated and that this phosphorylation is required for receptor internalization, but it is uncertain whether phosphorylation of the first only or both the first and third intracellular loops is necessary (Nakamura et al., 1998).

1.3.3.3 **The $NP(X)_{2,3}$ Y motif of the seventh transmembrane domain**

The highly conserved Tyr residue in this motif in TM7 of $\alpha 1_B$-adrenergic (Wang, J. et al., 1997), β_2-adrenergic (Barak et al., 1994; Ferguson et al., 1995), and GnRH (Arora et al., 1996) receptors was shown to be important for internalization. However, this motif is not required for internalization of the gastrin-releasing peptide

(Slice et al., 1994) and AT_{1A} (Hunyady et al., 1994c, 1995; Thomas et al., 1995b; Laporte et al., 1996) receptors, suggesting that this sequence does not represent a universal internalization signal for GPCRs.

Interestingly, the rapid internalization of a non-GPCR receptor, the low density lipoprotein receptor, requires a tetrapeptide sequence motif similar to the $NP(X)_{2,3}Y$ motif of GPCRs. The NPXY sequence, which is conserved in low density lipoprotein receptors from six species and in two other members of the low density lipoprotein receptor gene family, is required for their rapid internalization. Furthermore, this motif is also found in the cytoplasmic domains of the epidermal growth factor and insulin receptors, suggesting that it may play a role in internalization of these receptors (Trowbridge, 1991).

1.3.3.4 **The cytoplasmic C-terminal tail**

Reports demonstrating a role for the cytoplasmic C-terminal tail in agonist-promoted internalization of GPCRs are numerous (see Table 3). Elements of the cytoplasmic tails of the AT_{1A} (Hunyady et al., 1994b; Chaki et al., 1994; Balmforth et al., 1995; Thomas et al., 1995; Conchon et al., 1998), human B1 and B2 kinin (Faussner et al., 1998), β_2-adrenergic (Campbell et al., 1991; Hausdorff et al., 1991; Gabilondo et al., 1997), bombesin (Tseng et al., 1995); cholesystokinin-A (Go et al., 1998), gastrin-releasing peptide (Benya et al., 1993), glucagon-like peptide-1 (Widmann et al., 1997), Histamine H2 (Fukushima et al., 1997), interleukin-8 (Prado et al., 1996), lutropin/choriogonadotropin (Rodriguez et al., 1992; Wang et al., 1997; Lazari et al., 1998), neurotensin (Chalbry et al., 1995; Hermans et al., 1996), opioid receptor family (Trapaidze et al., 1996; Chu et al., 1997; Koch et al., 1998), parathyroid hormone (Huang et al., 1995), platelet-activating factor (Le Gouill et al., 1997; Ishii et al., 1998), thrombin (Shapiro et al., 1996), and thyrotropin-releasing hormone (Nussenzveig et al., 1993a; Ashworth et al., 1995) receptors, have been implicated in mediating their agonist-promoted internalization.

TABLE 3 STUDIES DEMONSTRATING A ROLE FOR THE CYTOPLASMIC C-TERMINAL TAIL IN GPCR INTERNALIZATION

Receptor	Reference
AT_{1A}	Hunyady et al., 1994b; Chaki et al., 1994; Balmforth et al., 1995 Thomas et al., 1995a,b; Conchon et al., 1998
human B1 and B2 kinin	Faussner et al., 1998
β_2-adrenergic	Campbell et al., 1991; Hausdorff et al., 1991; Gabilondo et al., 1997
bombesin	Tseng et al., 1995
cholesystokinin-A	Go et al., 1998
gastrin-releasing peptide	Benya et al., 1993
glucagon-like peptide-1	Widmann et al., 1997
Histamine H2	Fukushima et al., 1997
interleukin-8	Prado et al., 1996
lutropin/choriogonadotropin	Rodriguez et al., 1992; Wang et al., 1997; Lazari et al., 1998
neurotensin	Chalbry et al., 1995; Hermans et al., 1996
opioid receptor family	Trapaidze et al., 1996; Chu et al., 1997; Koch et al., 1998
parathyroid hormone	Huang et al., 1995
platelet-activating factor	Le Gouill et al., 1997; Ishii et al., 1998
thrombin	Shapiro et al., 1996
thyrotropin-releasing hormone	Nussenzveig et al., 1993a; Ashworth et al., 1995

1.3.4 **Function and biological role of agonist-promoted internalization**

Internalization of GPCRs into intracellular compartments following agonist exposure has potential relationships with other aspects of GPCR function. The possible relationships between receptor activation, receptor-G protein coupling, second messenger production, and agonist-promoted internalization are depicted schematically in Figure 2. In the next sections, the relationships of GPCR internalization to desensitization and resensitization are considered.

1.3.4.1 **Sequestration, downregulation and desensitization**

Internalization of GPCRs may contribute to desensitization as a result of the removal of receptors from the plasma membrane, thereby making them inaccessible to agonists (sequestration). Internalization was originally proposed to be a mechanism for desensitization based on the observation that the light membrane fraction containing internalized β_2-adrenergic receptor did not contain G_s (Waldo et al., 1983) Several studies have reported that pharmacological agents that inhibit internalization, but do not disrupt GPCR signaling, prevent desensitization (Yu et al., 1993; Pippig et al., 1995; Garland et al., 1996). In addition, when thyrotropin-releasing hormone receptor internalization was blocked by either overexpression of dominant-negative mutant dynamin, hypertonic sucrose solution, or the removal of the receptor's cytoplasmic tail, desensitization failed to occur for the truncated receptor only (Yu and Hinkle, 1998), suggesting that the cytoplasmic tail, which is required for receptor internalization, is additionally required for this receptor's desensitization. Perhaps cytoplasmic tail phosphorylation at Ser/Thr residues is a feature of both internalization and desensitization of the thyrotropin-releasing hormone receptor.

In contrast, a mutant β_2-adrenergic receptor and a cytoplasmic tail truncated platelet-activating factor receptor, both of which were defective for internalization, were shown to desensitize normally (Barak et al., 1994; Ishii et al.,1998). Similar results have been reported for m3 muscarinic and AT_{1A} receptors (Tobin et al., 1992; Thomas et al., 1995). For a number of GPCRs including the m2 muscarinic (Pals-Rylaarsdam et al., 1995), secretin (Holtmann et al., 1996), and neurokinin-1 (Garland et al., 1996) receptors it appears that internalization and desensitization are independent distinct processes. These results suggest that agonist-promoted internalization is an important mechanism of receptor desensitization in some GPCRs, while other receptors utilize desensitization mechanisms independent of internalization.

Internalization may contribute to downregulation if the internalized receptors are more rapidly degraded (Hertel et al., 1985). Downregulation is defined as a decrease in the total number of receptors in a cell, and has often been observed as a result of long-term agonist exposure. In two recent studies, it was reported that agonist-promoted internalization of the β_2-adrenergic (Gagnon et al., 1998) and the m2 muscarinic (Tsuga et al., 1998) receptors was partly responsible for influencing their downregulation. An earlier study by Hausdorff et al. (1991) showed that a β_2-adrenergic receptor mutant that was defective for internalization, displayed a wild type pattern of downregulation. These studies demonstrate that downregulation can be partly influenced by the internalization of receptors via the clathrin-coated pit endosomal pathway into lysosomes, where they are degraded.

1.3.4.2 **Resensitization**

It has been proposed that one of the roles for agonist-promoted internalization may be to mediate receptor dephosphorylation and resensitization (Yu et al., 1993; Barak et al., 1994; Garland et al., 1996). When β_2-adrenergic receptor internalization was inhibited by pharmacological treatments, such as hypertonic sucrose or concanavalin A, it was shown that both dephosphorylation and resensitization was prevented (Pippig et al., 1995). Other studies have also suggested a role for agonist-promoted internalization in accelerating the recovery from desensitization. Agonist-promoted internalization of the neurokinin-1 (Garland et al., 1996) and α_{1B}-adrenergic (Fonseca et al., 1995) receptor is critical for their resensitization. In addition, it was recently demonstrated that the cytoplasmic tail of one of the two isoforms of the rat μ-opioid receptor (the MOR1B isoform) facilitates its CCV-mediated endocytosis which, in turn, promotes accelerated receptor resensitization (Koch et al., 1998). Moreover, an internalization-defective platelet-activating factor receptor lacking its cytoplasmic tail, showed no evidence of resensitization (Ishii et

al., 1998). In contrast, a C-terminal cytoplasmic tail truncation mutant of the thyrotropin-releasing hormone receptor that displayed blocked agonist-promoted internalization, was able to resensitize at a normal rate (Yu and Hinkle, 1998).

1.3.5 **A model for agonist-promoted internalization**

Ferguson et al (1996b) incorporated the general conclusions of the literature reviewed above in a model for GPCR trafficking in response to the binding of an agonist (Figure 4).

Agonist binding results in activation of the receptor and consequent receptor-G protein coupling. Following the exchange of GDP for GTP on the G protein α subunit, the G_{α} and $G_{\beta\gamma}$ subunits dissociate leading to activation of the receptor's effector system. The receptor is then phosphorylated by GRKs. In the case of GRK2 and GRK3, $G_{\beta\gamma}$ is required to translocate the GRK to the plasma membrane. Receptor phosphorylation promotes the binding of arrestins, an event that targets the receptor for dynamin-dependent internalization via the clathrin-coated pit endosomal pathway where receptor dephosphorylation, release of receptor-bound arrestin and resensitization can occur. Acidification of the endosome leads to dissociation of the agonist (which can then undergo lysosomal degradation). The GPCR is then transported back to the cell surface where it is again able to bind agonist (Ferguson et al., 1996b).

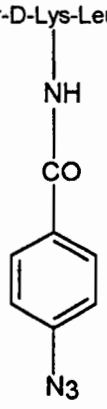
1.4 THE GONADOTROPIN-RELEASING HORMONE RECEPTOR

1.4.1 Introduction

The hypothalamic decapeptide GnRH (for structure see Table 4) is the central regulator of the reproductive system, acting via receptors on pituitary gonadotrope cells. GnRH receptors from mouse, rat, human, sheep and cow are members of the rhodopsin-like family of GPCRs (Reinhart et al., 1992; Tsutsumi et al., 1992; Kakar et al., 1993; Kaiser et al., 1992; Chi et al., 1993; Illing et al., 1993) and are coupled via $G_{q/11}$ to activation of phospholipase C, leading to calcium signaling (McArdle et al., 1992; Stojilkovic and Catt, 1992; Tse and Hille, 1992; Stojilkovic et al., 1994; Stojilkovic and Catt, 1995; Sealfon et al., 1997) and the release of gonadotropins by regulated exocytosis (van der Merwe et al., 1989; Davidson et al., 1991; Tse et al., 1993).

The mammalian GnRH receptor has a number of unique structural features (Figure 5). These include the presence of an unusually long and highly basic first intracellular loop, a reversed arrangement of the highly conserved Asp of TM2 and Asn of TM7 (Zhou et al., 1994), replacement of Tyr for Ser in the highly conserved DRY motif at the cytoplasmic end of TM3, and in the case of mammalian GnRH receptors, the absence of a C-terminal cytoplasmic tail. In contrast, GnRH receptors in non-mammalian species such as the catfish (Tensen et al., 1997), goldfish, frog, and chicken (Troskie et al., 1997) possess cytoplasmic tails like all other GPCRs (Figure 6).

TABLE 4 PRIMARY STRUCTURE OF GnRH ANALOGS USED IN THIS THESIS

Analog	Sequence
Mammalian GnRH	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-$GlyNH_2$
[D-Ala^6-*N*-$MeLeu^7$, Pro^9-NEt]GnRH	pGlu-His-Trp-Ser-Tyr-D-Ala-N-MeLeu-Arg-ProNEt
[His^5, D-Tyr^6]GnRH	pGlu-His-Trp-Ser-His-D-Tyr-Leu-Arg-Pro-$GlyNH_2$
Antagonist 26	Ac-D-p-ClPhe-Ac-D-p-ClPhe-D-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-$AlaNH_2$
[*N*-azidobenzoyl-D-Lys^6]GnRH	pGlu-His-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-GlyNH 2 

1.4.2 **Agonist-promoted internalization of the GnRH receptor**

The first demonstration of agonist-promoted internalization of the GnRH receptor was that of Hopkins and Gregory (1977), who utilized a GnRH analog coupled to ferritin. When dissociated pituitary cells were incubated with the analog, an even distribution of ferritin particles over the gonadotrope cell surface was observed under the electron microscope. After 15 min of incubation, the bound conjugates became aggregated and then formed clusters, which were subsequently internalized by endocytosis. Later, a biologically active rhodamine derivative of [D-Lys^6]GnRH was used to study the uptake of the fluorescent peptide by pituitary cells (Hazum et al., 1980; Naor et al., 1981). It was demonstrated that at first, the fluorescence was

evenly distributed over the cell surface. After 20 min of incubation, the rhodamine-GnRH tracer localized to fluorescent aggregates at one pole of the pituitary cells, a similar observation to that of Hopkins and Gregory (1977). Internalization of the fluorescent tracer became evident after 30 min. Similar observations were reported by Childs et al. (1983) with the use of a [biotinyl-D-Lys^6]GnRH derivative.

Pelletier et al. (1982) and Duello et al. (1983) utilized autoradiography to examine the fate of radioiodinated GnRH agonist following internalization, and demonstrated that the peptide was delivered to the Golgi region and lysosomes by vesicular transport. Utilizing [D-Lys^6]GnRH coupled to electron-opaque markers (colloidal gold or ferritin), Jennes et al. (1983) demonstrated that following internalization, the derivatives were routed to lysomes either directly or after passage through the Golgi apparatus. A later study compared the route and rate of internalization of GnRH in granulosa and pituitary cells (Childs et al., 1986). The GnRH receptor was localized with [biotinyl-D-Lys^6]GnRH, followed by avidin-gold or avidin-biotin-peroxidase complex staining. Binding and internalization of the [biotinyl-D-Lys^6]GnRH occurred rapidly for both cell types, while the initial processing of the peptide subsequent to internalization appeared to be different, with the [biotinyl-D-Lys^6]GnRH being rapidly transferred to the Golgi region in gonadotrope cells, suggesting a pathway for receptor recycling. In contrast, in granulosa cells the processing appeared to proceed from vesicles to endosomes, and only much later to the Golgi region (Childs et al., 1986).

The development of photoaffinity labeling provided an important experimental tool for the detection and isolation of membrane components. A photoaffinity derivative of GnRH, [*N*-azidobenzoyl-D-Lys^6]GnRH (see Table 4) (Hazum, 1981a, b), was used for GnRH receptor localization studies (Hazum et al., 1982a, b, 1984, 1985, Schvartz and Hazum, 1987). The major advantage of this approach was that the covalent attachment of the hormone to the receptor following photolysis allowed detection of the receptor-hormone complex throughout the cell. When dispersed rat pituitary cells

were incubated with ^{125}I-[*N*-azidobenzoyl-D-Lys^{6}]GnRH for 90 min at 4°C and subsequently photolyzed, most of the label remained associated with the cell surface membrane, while incubation for various time periods at 37°C followed by photolysis resulted in internalization of the derivative (Hazum et al., 1982a, b).

The photoaffinity labeling of GnRH receptors of plasma membrane preparations from rat pituitary cells resulted in the identification of a single specific band with an apparent molecular weight of 60 kDa (Hazum, 1981b). Later photoaffinity labeling studies (Hazum and Nimrod, 1982a, Hazum and Keinan, 1984, Iwashita and Catt, 1985) have demonstrated that GnRH receptors in the ovary and the testis, differ in size from that of the pituitary. The differences in size are due to different degrees of glycosylation in various cell types and species of mammal (Davidson et al., 1995, 1996, 1997).

Schvartz and Hazum (1987) used the [*N*-azidobenzoyl-D-Lys^{6}]GnRH derivative to follow the intracellular pathways of GnRH receptors subsequent to their internalization. In that study (Schvartz and Hazum, 1987), the site for GnRH receptor degradation appeared to be lysosomes, since pre-treatment with the lysosomotropic agents, chloroquine and methylamine, resulted in decreased receptor degradation. Receptor recycling was demonstrated on the basis of results obtained following treatment of the photaffinity labeled GnRH receptors with trypsin. A proteolytic fragment, characteristic of cell surface receptors, was obtained at all time-points measured, suggesting a rapid recycling pathway. It was postulated that the pathway of GnRH receptor recycling could involve passage through the Golgi apparatus and then via secretory granules (Hazum et al., 1985, Schvartz and Hazum, 1987).

Childs et al. (1986) compared the processing of GnRH in pituitary and granulosa cells (see above) showing that the primary point of processing in pituitary cells was the Golgi region, and also suggested that receptor recycling could proceed via secretory granules. Furthermore, based on the ineffectiveness of weak bases and a

proton ionophore (monensin) on GnRH receptor recycling, it was concluded that acidification of intracellular compartments was not essential for GnRH receptor recycling (Schvartz and Hazum, 1987). This result suggests also that GnRH dissociation from the receptor was not necessary for recycling to proceed. This is in contrast to the more classical recycling receptors such as those for low density lipoprotein and transferrin (Goldstein et al., 1985) which require endosome acidification for ligand-receptor dissociation, and subsequent receptor recycling.

The requirement for GnRH receptor activation for its internalization has been studied in some detail, with conflicting results. An early investigation into the internalization of GnRH receptor antagonists (Hazum et al., 1983) demonstrated that a rhodamine labeled antagonist showed a similar pattern of internalization to rhodamine-GnRH agonist studied previously (Hazum et al., 1980). In addition, the antagonist used was able to induce receptor clustering on the cell surface, as seen for agonists. In contrast, Loumaye et al. (1984) reported that a different antagonist to that used by Hazum et al. (1983), was not internalized, suggesting that activation of GnRH receptors by agonist binding is necessary to initiate receptor-mediated internalization. When the above two studies were repeated in an independent laboratory (Jennes et al., 1986), the results indicated that both antagonists were internalized by receptor-mediated internalization. It was suggested that the presence of antagonist in intracellular compartments could simply be related to the basal rate of GnRH receptor turnover in the plasma membrane. Additional reports supporting prior receptor activation as a requirement for GnRH receptor internalization have come from Suarez-Quian et al. (1986) and Wynn et al. (1986).

In the pituitary, it has been shown that internalization of GnRH receptors is not required for LH release, and that the system has a ongoing requirement for externally applied GnRH in order to sustain stimulated LH release (Conn and Hazum, 1981; Conn et al., 1981)

As reviewed in **Section 1.3.3**, most of the sequence motifs that influence the internalization of GPCRs are Ser/Thr-rich and are usually found in the third intracellular loop or C-terminal cytoplasmic tail of the receptor. Since mammalian GnRH receptors lack a cytoplasmic tail and Ser/Thr-rich regions in their intracellular loops, alternative motifs or residues must determine its agonist-promoted internalization. Amino acid residues occurring three regions of the GnRH receptor have been implicated in its internalization.

a) The highly conserved DRYXXV/IXXPL (*DRS*LA/TQ*PL* in the mouse GnRH receptor, see Figure 5) sequence of the second intracellular loop of GPCRs has been shown to be critical in agonist-promoted internalization of the mouse GnRH receptor (Arora et al., 1995, 1997). The replacement of Ser^{140} (Figure 5) with Tyr resulted in a increased internalization rate, while replacement of Leu^{147} (Figure 5) with either Ala or Asp markedly impaired internalization (Arora et al., 1995).

b) Two residues in the conserved $NP(X)_{2,3}Y$ sequence of TM7 of GPCRs, Asp^{318} and the aromatic moiety of Tyr^{322} (Figure 5) are also involved in the internalization of the GnRH receptor (Arora et al, 1996).

c) Ala^{261} (Figure 5) in the third intracellular loop of the GnRH receptor was recently reported to be critical for its agonist-promoted internalization (Myburgh et al., 1998).

1.4.3 **Desensitization of the GnRH receptor**

It is well established that pulsatile GnRH stimulation is necessary to produce a sustained output of gonadotropins from the mammalian pituitary *in vivo*, whereas continuous stimulation with GnRH agonist causes a cessation of gonadotropin secretion. This desensitization of the gonadotrope cell is of great interest as it is the basis for the clinical use of GnRH agonists to suppress the reproductive axis in a

number of human disorders (Millar et al., 1987). The mechanisms involved in desensitization of the gonadotrope have not been elucidated, and could in principle, operate at several levels including the GnRH receptor, G proteins, effector systems, gonadotropin synthesis and exocytosis.

As discussed in **Section 1.2**, phosphorylation of Ser/Thr residues within the cytoplasmic tail of a number of GPCRs influences their desensitization. The lack of a cytoplasmic C-terminal tail in mammalian GnRH receptors has prompted several investigations into GnRH receptor desensitization (Davidson et al., 1994; McArdle et al., 1995, 1996, Anderson et al., 1995, Willars et al., 1998). It has now been shown by several groups that the mammalian GnRH receptor itself does not undergo rapid homologous desensitization, suggesting that desensitization to GnRH occurs at a level beyond both the receptor and phospholipase C activation. When M3 muscarinic and GnRH receptors, both of which are coupled to phospholipase C, were co-expressed in αT3-1 cells and challenged with their respective agonists, only M3 muscarinic receptors underwent rapid desensitization (Willars et al., 1998), despite expression in the same cell background. This study demonstrates PLC-coupled GPCRs can undergo rapid desensitization in the αT3-1 cell line, and that the desensitization profiles are receptor-specific rather than cell-specific.

The GnRH receptor does contain consensus PKC phosphorylation sites (Tsutsumi et al., 1992) which could mediate heterologous desensitization, and phorbol ester was shown to attenuate GnRH-stimulated IP production (Davidson et al., 1994). However, IP production stimulated by direct activation of $G_{q/11}$ using GTPγS in permeabilized cells was also inhibited by phorbol ester, suggesting that the site of action of PKC on GnRH action lies distal to the GnRH receptor (Davidson et al., 1994).

McArdle et al. (1996) have demonstrated a novel form of desensitization to GnRH that is mediated at the level of mobilization of Ca^{2+} from intracellular pools, rather than an uncoupling of the receptor from its proximate effector system. A possible new mode of GnRH-induced desensitization, which is a brief pause (of about 10 min) in LH secretion at 40-50 min associated with a decrease in cell surface GnRH receptor binding sites, has been demonstrated by Cassina et al. (1997).

GnRH is known to downregulate GnRH receptors in pituitary cells by a number of mechanisms including reduction of receptor synthesis subsequent to a decrease in receptor mRNA levels (Mason et al., 1994), receptor degradation and endocytosis of the agonist-receptor complex (Stojilkovic et al., 1994). A recent study in which the cytoplasmic C-terminal tail of the catfish GnRH receptor was added to the C-terminus of the rat GnRH receptor, reported enhanced rat receptor downregulation when compared with the wild type rat GnRH receptor (Lin et al., 1998).

A potential role for GRKs and β-arrestins in the desensitization of the mammalian GnRH receptor was demonstrated in a recent study (Neill et al., 1998). Co-expression of GRK2 plus β-arrestin-2 suppressed GnRH-stimulated IP_3 production, with GRK3 plus β-arrestin-2 producing similar results. This is particularly interesting, as GRK phosphorylation sites in other GPCRs are usually located in the cytoplasmic C-terminal tail, which is absent in the mammalian GnRH receptor. Endogenous GRK2/3 and β-arrestin-1/2 expression was demonstrated in the rat anterior pituitary, suggesting the involvement of GRK-mediated regulation of the GnRH receptor *in vivo*. A member of a recently discovered family of proteins known as the regulators of G protein signaling (RGS3) has been reported to inhibit GnRH-stimulated IP_3 production, suggesting a role for RGS3 in GnRH receptor desensitization (Neill et al., 1997).

2 MATERIALS AND METHODS

2.1 MATERIALS

myo-[2-^{3}H]inositol and Na^{125}I were obtained from The Radiochemical Centre (Amersham, Bucks., UK). All other chemicals were obtained from Sigma Chemical Co (St. Louis, MO), Merck (Darmstadt, Germany), or BDH (Poole, England). [D-Lys^{6}]GnRH was a gift from Dr. R. Roeske, Department of Biochemistry and Molecular Biology, Indiana University, School of Medicine, Indiana, USA. Antagonist 26 was synthesized by Dr. David Coy, Tulane University School of Medicine, New Orleans, Louisiana, USA. mammalian GnRH, [D-Ala^{6}]GnRH and [His^{5}, D-Tyr^{6}]GnRH were synthesized by Mr. R.C. deL Milton from this laboratory.

2.2 CELL CULTURE

COS-1 cells were cultured in 150 cm^{2} tissue culture flasks (Corning, Cambridge, MA) in DMEM (Gibco, Grand Island, NY) containing 10% fetal calf serum (FCS)(Highveld Biological, Johannesburg, South Africa) in a 10% CO_2 atmosphere. For seeding of 12-well culture plates (Corning), DMEM was aspirated and the cells dispersed by the addition of 5 ml trypsin (BDH). Cells were pelleted at 400g, resuspended in 10 ml DMEM/10% FCS and counted using a haemocytometer.

For photoaffinity labeling studies, all experiments were performed on the αT3-1 gonadotrope-derived cell line (Windle et al., 1990). Cells were cultured in 150 cm^{2} tissue culture flasks in DMEM containing 10% FCS in a 10% CO_2 atmosphere, and seeded into 35 mm (10^{6} cells/dish) or 100 mm (3 X 10^{6} cells/dish) dishes (Corning).

2.3 SITE-DIRECTED MUTAGENESIS

2.3.1 Kunkel's Method

The mouse GnRH receptor cDNA (Tsutsumi et al., 1992) was subcloned between the EcoR1 and Xho1 sites of the phagemid pcDNA1/Amp (Invitrogen, San Diego, CA). Oligonucleotide-directed mutagenesis was performed using the method of Kunkel et al. (1991). After passage through E.coli dut⁻ung⁻F' strain CJ236, uridine-containing single-stranded DNA template was prepared using VCS M13 helper phage (Stratagene, La Jolla, CA). Mutant oligonucleotides (Table 5) were designed to encode the described amino acid substitutions as well as silent restriction sites to facilitate screening of mutant colonies. Oligonucleotides were phosphorylated with T4 polynucleotide kinase (Promega, Madison, WI), hybridized with the template, and second strand synthesis was completed using T7 DNA polymerase (Biorad, Richmond, CA) and T4 ligase (Biorad). Products of the reaction were used to transform competent E.coli strain XLI-blue cells. Plasmid DNA from ampicillin-resistant colonies was prepared using NucleoSpin miniprep spin columns (Macherey-Nagal, Düren, Germany) according to manufacturer's instructions and digested with the appropriate restriction enzyme and clones showing the additional silent restriction site were sequenced, confirming the mutations. DNA was sequenced manually by the dideoxy chain termination method (Sanger et al., 1977) using the Sequenase 2.0 DNA sequencing kit (United States Biochemical Corporation) according to manufacturer's instructions or by an automatic sequencer (Model 373A, Applied Biosystems).

2.3.2 PCR-based Site-Directed Mutagenesis

The chicken GnRH receptor cDNA (Troskie et al., 1997) was subcloned between the Not1 and Xho1 sites of the phagemid pcDNA1/Amp. For truncation mutations, two primers, pcDNA1/Amp vector specific sense primer T7; 5'- AAT ACG ACT CAC -3'

and chicken GnRH receptor DNA specific antisense primer encoding a stop codon as well as a silent restriction site (Table 5); were used to amplify the chicken GnRH receptor cDNA, deleting the tail DNA sequence. The PCR product was digested with Not1 and Xho1, ligated into pcDNA1/Amp and used to transform competent E.coli strain XL1-blue cells. Plasmid DNA from ampicillin-resistant colonies was sequenced, confirming the mutation. For point mutations, pcDNA1/Amp vector specific sense primer T7 and a chicken GnRH receptor DNA specific antisense primer (Table 5) and pcDNA1/Amp specific antisense primer SP6; 5'- ATT TAG GTG ACA CTA TAG -3' and a chicken GnRH receptor DNA specific sense primer (Table 5) encoding the described amino acid substitutions as well as silent restriction sites, were used to amplify the chicken GnRH receptor cDNA. The PCR products were digested with the appropriate enzymes, ligated into pcDNA1/Amp and used to transform competent E.coli strain XL1-blue cells. Plasmid DNA from ampicillin-resistant colonies was digested with the appropriate restriction enzyme and clones showing the additional silent restriction site were sequenced, confirming the mutations.

TABLE 5 PRIMERS USED FOR SITE-DIRECTED MUTAGENESIS

Mutation[a]	Primer Sequence
mD138A	5'- GAT TAG CCT GGC AAG ATC TCT GGC CAT CA -3'
mD138N	5'- GAT TAG CCT GAA CCG CTC CC -5'
mR139H	5'- TGG TGA TTA GTC TAG ACA AAT CCC TGG CC -3'
mR139K	5'- TGG TGA TTA GTC TAG ACC AAT CCC TGG CC -3'
mR139Q	5'- TGG TGA TTA GTC TAG ACC AAT CCC TGG CC -3'
cS337stop	5'- GTC ACT CGA GTC AAA TGG CTG CTT CAA TGC CC -3'
cS337A	5'- ATT GCC CAG CAC GTG AGA CAC AAA CCC ATC TC -3' 5'- GTC TCA CGT GCT GGG CAA TGG CTG CTT CAA TGC CC -3'
cS346stop	5'- AAG GCT CGA GTT AGA TGG GTT TGT GTC TCA C -3'
cT351stop	5'- AAG GCT CGA GTT ACT TCT CTG AGA CTG AGA T -3'
cD356stop	5'- AAG GCT CGA GTT ACC CAT CCT TGG TGG TCT -3'
cT362S363→AA	5' GAG GTG GCA GCA GGT GGA TCC AAT GGG ACA -3'
cS366stop	5'- AAG GCT CGA GTT AGC CAC CTG ATG TCA CCT -3'
cS366A	5'- GGC CAG GTG ACG TCA GGT GGC GCC AAT GGG -3'
cT369T370→AA	5'- TCA GGT GGA TCC AAT GGG GCA GCC GTT AAC -3'

[a] The species of GnRH receptor is indicated by the first (lowercase) letter. m=mouse, h=human, and c=chicken. The mutation is indicated using the single-letter amino acid code.

Example: mD138A refers to mutation of Asp^{138} to Ala in the mouse GnRH receptor

All mutant GnRH receptor cDNA constructs listed in the above table were produced by the author as part of this thesis, except for the following:

cS337stop, cS337A	John Lopes, our laboratory
hI143A, hI143L	Dr. Stuart Sealfon, Mount Sinai University, New York, USA

2.4 TRANSIENT TRANSFECTION

Plasmid DNA for transfection was prepared using Nucleobond AX columns (Macherey-Nagal, Düren, Germany) according to the manufacturer's instructions. One day prior to transfection, COS-1 cells were seeded into 12-well culture plates at a density of 75,000 cells per well. On the day of transfection cells were washed once with serum-free DMEM containing 10 mM HEPES, pH 7.4 (HEPES/DMEM) and incubated with 1.0 µg of plasmid DNA and 1.5 µl of Lipofectin (Gibco) in 0.5 ml of serum-free OPTI-MEM (Gibco) medium. 6 hrs later, 0.5 ml of DMEM with 20% FCS was added, and cells were allowed to grow for 48 hrs before radioligand binding, inositol phosphate, or internalization assays were performed.

2.5 MEASUREMENT OF RADIOLIGAND BINDING

Radioligand binding and internalization studies of the mouse GnRH receptor were carried out using [D-Ala^6]GnRH ([D-Ala^6-*N*-Me-Leu^7,Pro^9-NEt]GnRH)(agonist) and Antagonist 26 ([Ac-D-*p*-ClPhe^1,D-*p*-ClPhe^2,D-Trp^3,D-Lys^6,D-Ala^{10}-NH_2]GnRH) radioligands. For chicken GnRH receptor internalization studies, the agonist [His^5, D-Tyr^6]GnRH was used as radioligand (Flanagan et al., 1998). The peptides were iodinated with $Na^{125}I$ (Amersham) using chloramine T (Millar et al., 1984). Five µg of peptide was dissolved in 40 µl of solution containing 0.5 M phosphate buffer (0.5 M NaH_2PO_4 , pH 7.6). To this, 1 mCi (10 µl) $Na^{125}I$ was added, followed by the addition of 85 nmoles chloramine T in 10 µl phosphate buffer to start the iodination reaction. After 10 sec, 84 nmoles of sodium metabisulphite in 20 µl phosphate buffer was added to stop the iodination reaction. The resulting solution was purified on a reverse-phase C18 HPLC column, eluting with a 0-60% acetonitrile gradient in 0.01 M ammonium acetate for [D-Ala^6]GnRH (Figure 7) and 0-80% acetonitrile gradient in

0.01 M ammonium acetate for Antagonist 26 (Figure 8) and [His^5, D-Tyr^6]GnRH (Figure 9). 1.5 ml fractions were collected and peptide fraction stored at -70°C.

Radioligand binding was measured in triplicate wells using intact, transiently transfected COS-1 cells in 12-well culture plates. Cells were washed for 5-10 min in ice-cold Buffer A (140 mM NaCl; 4 mM KCl; 20 mM HEPES, pH 7.4; 8 mM D-glucose) containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, then incubated at 0°C (on ice) in 0.5 ml of the same buffer containing 10^5 cpm radioligand per well (approx. 50 pM) and varying concentrations of the appropriate unlabeled peptide for 3 hrs, followed by 5 rapid washes in ice-cold Buffer A containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.5% BSA (fatty acid free, Pentex fraction V, Miles Laboratories). Cells were solubilized in 0.5 ml of 0.1 N NaOH and radioactivity counted in a γ counter (RIAstar, Packard). Unless otherwise stated, non-specific binding was determined in parallel using untransfected COS-1 cells, and was subtracted at each dilution.

2.6 MEASUREMENT OF INOSITOL PHOSPHATE PRODUCTION

24 hrs after transfection, cells were labeled overnight with 2 μCi/ml myo-[2-^{3}H]inositol (Amersham) in 0.25 ml/well Medium 199 (Gibco) containing 5% FCS, washed twice for 5 min in Buffer A containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$ at 37°C, then stimulated with GnRH as required, in Buffer A containing 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 10 mM LiCl for 1 hr at 37°C with gentle agitation. Incubations were stopped after 1 hr with 0.5 ml perchloric acid (PCA) stopping solution (5 mM EDTA, 5 mM DTPA, and 3.6% PCA (v/v)) and 50 μl 2 mM phytic acid, neutralized with KOH, and total inositol phosphates chromatographed on Dowex 50X8-200 (Sigma) ion exchange columns, eluted with 3 ml 1 M ammonium formate; 0.1 M formic acid, and radioactivity determined (Davidson et al., 1990).

2.7 MEASUREMENT OF RECEPTOR INTERNALIZATION

2.7.1 Acid-Wash Method

Internalization of GnRH receptors was measured by a modification of the acid-wash method of Hazum et al. (1983a). Transfected COS-1 cells in 12-well culture plates were washed once with ice-cold HEPES/DMEM, then incubated at 0°C (on ice) in HEPES/DMEM with 10^5 cpm (approx. 50 pM) ^{125}I-[D-Ala^6]GnRH or ^{125}I-[His^5, D-Tyr^6]GnRH per well for 3 hrs and 5 hrs respectively. This pre-binding step was included to allow radioligand binding to reach equilibrium, under conditions where no internalization takes place. Cells were then warmed to 37°C in the original medium, and radioligand internalization was stopped after varying periods of time by placing the cells at 0°C and rapidly washing twice with ice-cold phosphate-buffered saline (PBS). Acid-sensitive bound radioligand, representing cell surface-bound label, was removed by the addition of 1 ml ice-cold acid solution (150 mM NaCl, 50 mM acetic acid, pH 2.8) for 10 min. After removal of the acid wash, cells were solubilized with 1 ml of 1 N NaOH to determine acid-resistant (internalized) radioligand content. Both acid-sensitive and -resistant fractions were counted. Non-specific binding was determined in parallel using untransfected COS-1 cells, and was subtracted at each time-point. Radioligand internalization was expressed as percent of total cell-associated label (cell surface-bound plus acid-resistant) at each time-point (see Equation 1). In all % INTERNALIZATION plots, the % internalization at t = 0 was subtracted from the % internalization values at the remaining time-points, so that all curves begin at 0% internalization at t = 0.

Equation 1:

$$\% \text{ internalization} = \frac{\text{acid-resistant cpm}}{\text{acid sensitive cpm + acid-resistant cpm}} \times 100$$

2.7.2 **Development of the 3, 3'-Diaminobenzidene cross-linking method for measuring GnRH receptor internalization**

Internalization of GnRH receptors was also measured by a modification of the horseradish peroxidase (HRP)-mediated 3, 3'-diaminobenzidine (DAB) cross-linking method described by Thilo et al. (1995). In this method, transfected COS-1 cells are allowed to internalize radioligand in the presence of HRP for varying periods. Internalization is stopped by washing the cells on ice. Extracellular HRP is also removed during these washes. The cells are then incubated on ice in the presence of DAB, which diffuses into intracellular compartments containing internalized radioligand and HRP. The enzymatic activity of HRP polymerizes DAB in endosomes with consequent cross-linking of endosomal content proteins (including radioligand-GnRH receptor complexes) to the DAB polymer. The cells are then solubilized in detergent, and the detergent insoluble cross-linked polymer (representing internalized radioligand-receptor complex) separated from soluble radioactivity (representing cell surface radioligand-receptor complex) by centrifugation. The radioactivity in the two fractions is determined, and the insoluble fraction expressed as % of total (soluble + insoluble) radioactivity.

Transfected COS-1 cells in 12-well culture plates (Costar) were placed at 0°C (on ice), washed once with ice-cold HEPES/DMEM and then incubated at 0°C in HEPES/DMEM containing 1 mg/ml HRP (Sigma) with ^{125}I-[D-Ala^6]GnRH for 3 hrs. Cells were then warmed to 37°C for varying periods of time. Internalization was stopped by placing the cells at 0°C and HRP removed by washing twice with ice-cold PBS. Cells were then incubated for 30 min at 0°C in the dark with HEPES/DMEM containing 0.45 mg/ml DAB (Sigma) and 50 mM ascorbic acid (Merck), pH 7.0. Ascorbic acid was added to prevent DAB cross-linking of cell surface components due to residual plasma-membrane-associated HRP (Stoorvogel et al.,1996). Hydrogen peroxide was added to a final concentration of 0.005% (v/v) and cells were incubated for a further 30 min at room temperature in the dark. Cells were then

solubilized in 1% (v/v) Triton X-100 and insoluble material was pelleted by centrifugation at 10,000g for 10 min. Radioactivity in the supernatant and pellet, representing cell surface and internalized radioligand respectively, were counted, and internalized radioligand was expressed as a percentage of total bound label at each time-point. In all % INTERNALIZATION plots, the % internalization at t = 0 was subtracted from the % internalization values at the remaining time-points, so that all curves begin at 0% internalization at t = 0.

As controls, samples were kept at 0°C in the presence of HRP (no internalization), or H_2O_2 or DAB was omitted. In all cases, control solubility of radioligand in Triton X-100 was between 90 and 95%. Non-specific binding was determined in parallel using untransfected COS-1 cells, and was subtracted at each time-point.

2.8 PHOTOAFFINITY LABELING

2.8.1 Labeling procedure

The photoreactive GnRH analog, [*N*-azidobenzoyl-D-Lys^6]GnRH was synthesized by Dr. Daniel Assefa in our laboratory according to Hazum (1981a,b). Five μg of the photoreactive analog was iodinated using chloramine T as described in Section 2.5 and purified on a reverse-phase C18 HPLC column, eluting as a single radioactive peak with a 0-60% acetonitrile gradient. All the procedures prior to UV irradiation were performed in minimal lighting, conditions which had been shown not to cause photolysis of the photoreactive ligand. The iodinated ligand was stored at -70°C. Intact αT3-1 cells were incubated with 0.5 ml (35 mm dishes) or 1.2 ml (100 mm dishes) Buffer A containing 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10^6 cpm (35 mm dishes) or 5 X 10^6 cpm (100 mm dishes) of ^{125}I-[*N*-azidobenzoyl-D-Lys^6]GnRH at 4°C for 90 min. Buffer A consisted of 140 mM NaCl; 4 mM KCl; 20 mM HEPES, pH 7.4; 8 mM

D-glucose. The dishes (on ice) were irradiated for 90 sec at a distance of 2 cm from a Spectroline TR-312A UV transilluminator.

2.8.2 **Determination of the *in vitro* stability of the ^{125}I-[*N*-azidobenzoyl-D-Lys^6]GnRH cross-link to the GnRH receptor**

Photoaffinity labeled αT3-1 cells in 100 mm dishes were detached in ice-cold binding buffer (10 mM HEPES, 1 mM EDTA, pH 7.4), homogenized with 15 strokes of a glass Dounce homogenizer, and centrifuged for 10 min at 400g to pellet nuclei. The supernatant was centrifuged at 10000g for 30 min at 4°C, and the membrane pellet resuspended by drawing it through a fine needle in 0.25 ml of binding buffer per dish of cells. Following further manipulations (see Figure legends), Triton X-100 was added from a 10% stock solution to a final concentration of 1% and membranes solubilized by incubating on ice for 30 min. Unsolubilized material was removed by centrifugation at 10000g for 10 min, and the supernatant was mixed with SDS sample buffer and an aliquot electrophoresed for 18 hrs at room temperature on 10% SDS-polyacrylamide gels (Schagger and von Jagow, 1987). Gels were stained with Coomassie Blue (0.1% Coomassie Brilliant Blue; 40% methanol; 10% acetic acid) and autoradiographed using Kodak X-OMAT autoradiography film by exposing for 2-14 days at -70°C. Molecular masses of labeled bands were determined by graphical interpolation from plots of R_f vs. log molecular weight for standard proteins.

2.8.3 **Intracellular degradation of the ^{125}I-[*N*-azidobenzoyl-D-Lys^6]GnRH cross-linked GnRH receptor**

Immediately following UV irradiation, photoaffinity labeled αT3-1 cells in 35 mm dishes were washed twice with ice-cold PBS/1% BSA followed by one wash with ice-cold PBS. 1.5 ml HEPES/DMEM was added to all dishes which were then incubated at 37°C for the indicated times. At each time-point the dishes were transferred onto

ice, the medium collected in tubes and counted in a γ-counter. The cells were detached in ice-cold binding buffer (10 mM HEPES; 1 mM EDTA, pH 7.4), transferred to 1.5 ml eppendorff tubes, and pelleted at 400g for 10 min. The pellet was solubilized in 1 X RIPA buffer (150 mM NaCl; 50 mM Tris, pH 8.0; 5 mM EDTA; 1% Nonidet P-40; 0.5% sodium deoxycholate; 0.1% SDS) and unsolubilized material removed by centrifugation at 10000g for 10 min. The supernatant was mixed with SDS sample buffer and electrophoresed as described above.

2.9 DATA PRESENTATION

Data points and error bars represent the SEM of duplicate or triplicate determinations. The absence of error bars indicates that the error was smaller than the dimensions of the symbol. Peptide concentrations required to stimulate half-maximal IP production (EC_{50}) were estimated by non-linear regression to equation 2, where IP is the IP response to concentration A of agonist, and IP_{max} is the maximal response, using Prism (Graphpad) software. Peptide concentrations required to half-maximally inhibit binding of radioligands (IC_{50} values) were estimated by non-linear regression to the single-site binding equation with unitary Hill coefficient (see Equation 3). K_i values were obtained by correction of the IC_{50} values by the method of Munson and Rodbard (1988). Internalization data were fitted by non-linear regression to the single component exponential equation (see Equation 4, and Section 2.9.1 below for derivation), where Y_{max} is the maximal % internalization, K the rate constant, and t the time.

Equation 2:

$$IP = \frac{IP_{max}}{(1 + EC_{50}/A)}$$

Equation 3:

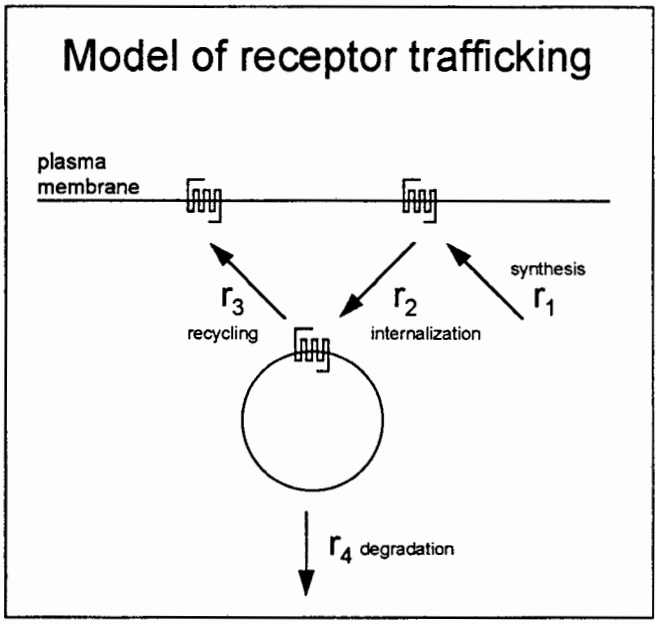
$$Y = Y_{min} + \frac{(Y_{max} - Y_{min})}{(1 + 10^{(X - \log IC50)})}$$

X = log(peptide concentration)

Equation 4:

$$F = F_{max}(1 - e^{-\lambda t})$$

2.9.1 **Derivation of a simple model for receptor internalization (Equation 4)**



Let:
s = number of surface receptors
i = number of internalized receptors

r_1 = rate of delivery of new receptors to the surface
r_2 = rate of internalization of receptors
r_3 = rate of recycling of receptors
r_4 = rate of degradation of receptors

Assumptions of the model

1. We treat only the simple case where r_2 , r_3 >> r_1 , r_4 , and consider only the larger receptor fluxes r_2 and r_3
2. The total number of receptors is assumed to be constant, T = s + i
3. We assume first-order kinetics for internalization and recycling rates,

i.e. $r_2 = k_2 s$
$r_3 = k_3 i$

Since r_4 is neglected, we may write the differential equation

$$di/dt = r_2 - r_3 = k_2 s - k_3 i = k_2 T - i(k_2 + k_3)$$

Let the fraction of internalized receptors, $F = i/T$

Then,

$dF/dt = d(i/T) / dt = 1/T(di/dt) = k_2 - F(k_2 + k_3)$,from which we obtain,

$$dF/dt = (k_2 + k_3)[k_2 / (k_2 + k_3) - F] \quad \textbf{(eqn. 5)}$$

which is of the form $dY/dt = b(a - Y)$, having the integral $Y = a(1 - e^{-bt})$

Therefore, integrating eqn. 5 yields,

$$F = [k_2 / (k_2 + k_3)] [1 - e^{-(k2+k3)t}] \quad \textbf{(eqn. 6)}$$

At steady state ($t = \infty$), the internalized fraction F is maximal, so that

$F_{max} = k_2 / (k_2 + k_3)$

Eqn. 6 may then be written

$$F = F_{max} (1 - e^{-\lambda t}) \quad \textbf{(eqn. 4)}$$

If experimental receptor internalization data is fitted to eqn. 4, the rate constants k_2 **(internalization rate constant)** and k_3 **(recycling rate constant)** are obtained from the relations,

$$k_2 = \lambda F_{max} \quad \textbf{(eqn. 7)}$$

$$k_3 = \lambda(1 - F_{max}) \quad \textbf{(eqn. 8)}$$

If F_{max} is expressed as % of total cell-associated receptors, then

$$k_2 = \lambda F_{max} \quad \textbf{(eqn. 9)}$$

$$k_3 = \lambda(100 - F_{max}) \quad \textbf{(eqn. 10)}$$

3 ROLES OF THE HIGHLY CONSERVED Asp^{138}-Arg^{139} PAIR IN ACTIVATION, COUPLING, AND AGONIST-PROMOTED INTERNALIZATION OF THE GnRH RECEPTOR

3.1 RESULTS

To investigate the role of Asp^{138} and Arg^{139}, the following GnRH receptor mutations were constructed: mD138N, mD138A, mR139Q, mR139K, and mR139H. B_{max}, K_d and K_i values for the mD138N and mR139Q receptors are given in Table 6.

3.1.1 The mR139Q mutation

The mR139Q receptor displayed 8-fold lower binding affinities for GnRH and Antagonist 26, and a 2-fold lower affinity for the superagonist [D-Ala^6]GnRH (Table 6, Figures 10-12). However, this mutant receptor exhibited increased binding (B_o) of both GnRH agonist and antagonist radioligands, an unusual combination of findings which implies increased receptor number. Calculated receptor numbers (B_{max}) using K_d values derived from homologous displacement binding experiments revealed that the mR139Q GnRH receptor was overexpressed by 6- to 10-fold, irrespective of whether binding was measured using agonist or antagonist radioligand (Table 6). As binding was measured in intact cells, the values for receptor numbers represent receptors expressed on the cell surface, and not intracellular pools of receptors.

Although the mR139Q GnRH receptor was expressed at higher levels than wild type, maximal GnRH-stimulated IP production in the mR139Q GnRH receptor-expressing cells was only 68±7.0% of wild type (Figure 13 and Table 7). In addition, the dose-response curve for IP production mediated by the mR139Q GnRH receptor was markedly shifted to the right, with a 150-fold higher EC_{50} than the wild type receptor (Figure 13 and Table 7). Although part of this increase in EC_{50} can be attributed to

the 8-fold decrease in affinity of the mutant receptor for GnRH (Table 6), it was evident that the mutant receptor has, in addition, a markedly lower coupling efficiency to its effector system.

TABLE 6 AGONIST AND ANTAGONIST BINDING PARAMETERS FOR MOUSE WILD TYPE AND MUTANT GnRH RECEPTORS

	Wild type	mD138N	mR139Q
Agonist binding			
B_{max} (% of WT)[a]	100	55.9±5.9 (5)	627±108 (5)
K_d ([D-Ala^6]GnRH)(nM)	0.36±0.07 (3)	0.37±0.08 (3)	0.77±0.12 (3)
K_i (GnRH)(nM)	1.09±0.35 (2)	1.38±0.35 (2)	8.88±0.98 (2)
Antagonist binding			
Bmax (% of WT)[b]	100	67.3±15.2 (2)	997±187 (2)
K_d (nM)	0.087±0.017 (2)	0.065±0.004 (2)	0.704±0.024 (2)

Values shown are mean ± S.E.M. of the number of independent experiments shown in parentheses.

[a] Total receptor number (B_{max}) determined utilizing agonist radioligand ^{125}I-[D-Ala^6]GnRH, calculated using K_d values derived by homologous displacement with unlabeled peptide.

[b] Total receptor number (B_{max}) determined utilizing antagonist radioligand ^{125}I-Antagonist 26, calculated using K_d values derived by homologous displacement with unlabeled peptide.

Since the maximal IP response (IP_{max}) is dependent on receptor number as well as coupling efficiency, and potency (EC_{50}) for second messenger production depends on both binding affinity (K_i) and receptor number (B_{max}), a quantitative definition of coupling efficiency is required to compare receptor mutants which also have altered levels of expression and/or agonist binding affinity. An operational quantitative measure of coupling efficiency (ε) may be arbitrarily defined as

$\varepsilon=(IP_{max}/B_{max})/(EC_{50}/Ki)$ and normalized to a value of ε = 100% for the wild type receptor. Defined in this way, receptor mutants with impaired coupling are characterized by ε values less than 100% (Ballesteros et al., 1998). The mR139Q GnRH receptor had a coupling efficiency ε = 6%, indicating severe uncoupling.

TABLE 7 INOSITOL PHOSPHATE PRODUCTION PARAMETERS FOR MOUSE WILD TYPE AND MUTANT GnRH RECEPTORS

	Wild type	mD138N	mR139Q
Inositol phosphate production			
IP_{max} (% WT)	100	98±6.0 (2)	68±7.0 (3)
EC_{50} (GnRH)(nM)	0.44±0.19 (4)	0.31±0.06 (3)	65.2±7.0 (3)
Coupling efficiency[a] (ε) (% WT)	100	310	6

Values shown are mean ± S.E.M. of the number of independent experiments shown in parentheses.

[a] ε is an arbitrary measure of coupling efficiency, defined as $\varepsilon = (IP_{max} / B_{max}) / (EC_{50} / K_i)$, normalized to a value of 100% for the wild type receptor (Ballesteros et al., 1998). The value given was calculated using the mean IP_{max}, K_i, and EC_{50} values for GnRH, and the mean B_{max} value from binding experiments using agonist radioligand.

3.1.2 **The mD138N mutation**

The mD138N GnRH receptor had essentially unchanged agonist and antagonist binding affinities, but its expression was decreased to 55% and 67% of wild type level, measured using agonist and antagonist radioligands respectively (Figures 10, 11, and 12, Table 6). GnRH-stimulated IP production parameters were similar to wild type, indicating that the mD138N GnRH receptor is well coupled to its effector system (Figure 13, Table 7). Furthermore, the fact that the mD138N GnRH receptor showed IP_{max} and EC_{50} values similar to the wild type receptor, despite its reduced

expression level, indicated that the mD138N mutation enhanced the coupling efficiency of the receptor, as reflected in its increased coupling efficiency (ε) value of 310% (Table 7).

3.1.3 **The mR139K, mR139H, and mD138A mutations**

The mR139K, mR139H, and mD138A mutations completely abolished agonist (Figure 14) and antagonist (Figure 15) binding, as well as IP production (Figure 16), indicating either gross misfolding, or failure of expression of these mutant receptors.

3.1.4 **Temperature modulation of agonist binding**

Some GPCRs, including the α_1- and β_2-adrenergic receptors exhibit a low-temperature-induced increase in agonist binding affinity, which has been suggested to be similar to the transition from a resting low-affinity state (R) to an activated high-affinity state (R*) (Weiland et al., 1979; Schwarz et al., 1986a, b; Lynch et al., 1988) The wild type mouse GnRH receptor also showed temperature modulation of agonist binding which was most clearly demonstrated by rapid dissociation of radioligand bound at 4°C upon shifting the receptor-ligand complex to 37°C. In Figure 17, wild type mouse GnRH receptors were allowed to bind radioligand at either 4°C or 37°C, followed by a shift to the opposite temperature for the same incubation time. At various time-points during these incubation periods the total amount of radioligand bound was measured. Rapid dissociation of radioligand bound at 4°C was observed following the shift to 37°C (Figure 17). In addition, a shift from 37°C to 4°C produced the opposite result, i.e. increased binding of agonist (Figure 17).

Since $Arg^{3.50}$ was essential for coupling, it was of interest to determine whether this residue had an essential role in the transition between the high and low agonist affinity states induced by low temperature. Put another way, since the mR139Q

mutant receptor was poorly coupled (see **Section 3.1.1**) it was of interest to determine whether it could achieve the high agonist affinity (R*) state induced by low temperature. Figure 18 shows the dissociation of radioligand bound at 4°C following a shift to 37°C, in the wild type GnRH receptor and in the mD138N and mR139Q mutant receptors. Both mutant receptors exhibited marked loss of ligand binding at the higher temperature, similar to the wild type receptor.

3.1.5 **Optimization of the acid-wash method for measuring GnRH receptor internalization**

The time-course of binding of ^{125}I-[D-Ala^6]GnRH to wild type mouse GnRH receptors (Figure 19) shows that binding equilibrium was approached after 3 hrs at 0°C, when 10^5 cpm ^{125}I-[D-Ala^6]GnRH is added per well. A 3 hr pre-incubation at 0°C was therefore used for further internalization studies.

The length and number of acid washes required to maximally remove surface-bound ^{125}I-[D-Ala^6]GnRH (acid-sensitive fraction) was determined (Figure 20). One 10 min acid-wash was found to remove more than 90% of surface-bound radioligand, while two 10 min or one 30 min acid-wash did not increase this fraction. One 10 min acid-wash was therefore used for further internalization studies.

3.1.6 **Optimization of the 3, 3'-Diaminobenzidene cross-linking method for measuring GnRH receptor internalization**

The time-course of binding of ^{125}I-Antagonist 26 to wild type mouse GnRH receptors (Figure 21) shows that binding equilibrium was not reached after 4 hrs at 0°C.

When the acid-wash method was used to measure internalization of ^{125}I-Antagonist 26, it was found that the acid solution was unable to remove more than 50% of the

surface-bound radioligand (Figure 22). For this reason it was necessary to develop the DAB cross-linking method for studying the internalization of antagonist-occupied GnRH receptors.

3.1.7 **Internalization of antagonist-occupied mouse wild type GnRH receptors**

The DAB cross-linking method was used to evaluate the internalization of the antagonist radioligand, ^{125}I-Antagonist 26 (Figure 23 left) by the wild type mouse GnRH receptor. Surface binding increased linearly with time and did not plateau during the period measured (Figure 23, left top panel). This slow on-rate is also reflected in the time-course of binding of ^{125}I-Antagonist 26 at 4°C (Figure 21). The fraction of bound antagonist radioligand that became internalized in 90 min was very small (Figure 23, left bottom panel). In contrast, more than 30% of agonist radioligand bound to receptors was internalized in 90 min (Figure 23 right panels).

3.1.8 **Validation of the acid-wash method for measuring agonist-occupied GnRH receptor internalization**

Untransfected cells were used as a control to determine whether the internalization of agonist radioligand in transfected COS-1 cells was entirely receptor-mediated, or whether non-specific bulk-phase endocytosis contributed to internalization.

As shown in Figure 24, acid-resistant ('internalized') agonist radioligand remained constant with time in untransfected cells, indicating that bulk-phase endocytosis does not contribute significantly to internalization of radioligand using this protocol. In all subsequent experiments, non-specific acid-resistant and acid-sensitive radioactivity was determined at each time-point in untransfected COS-1 cells in parallel and subtracted from the data. A component of the non-specifically surface-bound radioligand decreased after warming to 37°C.

3.1.9 **Agonist-promoted internalization of the mD138N and mR139Q mutant GnRH receptors**

The role of Arg^{139} in agonist-promoted internalization of the GnRH receptor was evaluated by determining the kinetics of receptor-mediated ^{125}I-[D-Ala^6]GnRH internalization using both the acid-wash method and DAB cross-linking method (Figures 25 and 26). Both methods gave similar results. After 90 min incubation at 37°C, 34-40% of bound label was internalized by the wild type receptor, while the mR139Q mutant receptor exhibited markedly decreased internalization (38-52% of wild type level, Table 8). The mD138N mutant consistently showed slightly enhanced internalization (117-121% of wild type level, Table 8).

The % internalization data were fitted to equation 4 (**Section 2.9.1**) by non-linear regression, which yielded values for F_{max} and λ. The internalization rate constants (k_2) and the recycling rate constants (k_3) were derived using equations 9 and 10 (**Section 2.9.1**).

The mR139Q receptor displayed a decreased internalization rate constant (k_2) and increased recycling rate constant (k_3) (Table 8). Both rate constants for the mD138N receptor were similar to wild type (Table 8).

TABLE 8 AGONIST-PROMOTED INTERNALIZATION PARAMETERS FOR MOUSE WILD TYPE AND MUTANT GnRH RECEPTORS

	Wild type	mD138N	mR139Q
Acid-wash method			
% WT[a]	100	117±9.9 (3)	51.5±2.3 (3)
F_{MAX}[b]	39.8±3.2	46.4±4.0	20.5±4.0
λ[c]	0.031±0.006	0.037±0.009	0.045±0.006
k_2[d]	1.24	1.69	0.92
k_3[e]	1.88	1.95	3.57
DAB cross-linking method			
% WT[a]	100	121±5.7 (3)	37.8±3.0 (3)
F_{MAX}[b]	34.5±1.8	41.9±2.0	13.3±0.6
λ[c]	0.042±0.006	0.043±0.006	0.045±0.006
k_2[d]	1.44	1.78	0.60
k_3[e]	2.74	2.47	3.89

[a] Internalization of ^{125}I-[D-Ala^6]GnRH after 90 min at 37°C, normalized to % of wild type. Internalization was measured using the both acid-wash and DAB cross-linking methods (see **Section 2.7**). Values shown are mean ± S.E.M. of the number of independent experiments shown in parentheses.

[b] F_{MAX} , the extrapolated maximal % internalization at equilibrium, was determined by curve fitting.

[c] λ, the time constant, determined by curve fitting.

[d] k_2, the internalization rate constant in %.min^{-1}, was calculated from equation 9, **Section 2.9.1**

[e] k_3, the recycling rate constant in %.min^{-1}, was calculated from equation 10, **Section 2.9.1**

3.1.10 **The role of PKC activation in agonist-promoted internalization**

Since the mR139Q receptor was uncoupled, its defective internalization could have resulted from its failure to activate signaling pathways. To determine whether the impaired internalization of the mR139Q GnRH receptor was due to its inability to activate phospholipase C and hence PKC, cells expressing wild type and mR139Q

GnRH receptors were pre-incubated with the phorbol ester PMA before determination of radioligand internalization. PMA increased wild type receptor internalization at 90 min from 33.3±2.2% to 39.0±1.8%, and increased mR139Q GnRH receptor internalization from 13.1±0.7% to 20.6±1.6% (Figure 27).

3.1.11 **The requirement for an intact arginine-cage for agonist-promoted internalization**

The requirement for an intact Arg-cage (see **Section 1.3.3.1**) for efficient agonist-promoted internalization was evaluated by determining the kinetics of receptor-mediated ^{125}I-[D-Ala^6]GnRH internalization by hI143A and hI143L mutant receptors. Figure 28 shows the time-course of internalization of ^{125}I-[D-Ala^6]GnRH in COS-1 cells expressing wild type human GnRH receptor, hI143A and hI143L mutant GnRH receptors. After 90 min incubation at 37°C, the wild type receptor internalized 25.3±1.7% of bound label, while the hI143A mutant receptor exhibited markedly decreased internalization (46.3±4.3% of wild type level). The hI143L mutant GnRH receptor showed a level of internalization similar to wild type (88.4±1.0% of wild type level).

3.2 **DISCUSSION**

3.2.1 **Role of Arg^{139} in coupling**

The arginine residue (Arg$^{3.50}$) located at the interface between the third transmembrane domain and the cytoplasm is conserved in all members of the rhodopsin-like subgroup of GPCRs, and is nearly always preceded by an acidic residue (Asp or Glu). This (E)DRY motif has been proposed to play an essential role in the coupling of these receptors to G proteins (Franke et al., 1990, 1992; Ohyama et al., 1992; Min et al., 1993; Rosenthal et al., 1993; Benya et al., 1994; Zhu et al.,

1994; Jones et al., 1995; Dhanwada et al., 1996). In the present study, the role of these residues, Asp^{138} and Arg^{139}, in coupling and agonist-promoted internalization of the mouse GnRH receptor was investigated by mutating them to neutral amide residues.

The mR139Q substitution resulted in decreased binding affinities for both GnRH agonist and antagonist, increased cell surface receptor number and a markedly increased EC_{50} of GnRH for IP production. The mR139Q GnRH receptor was severely uncoupled since the IP_{max} in mR139Q transfected cells was lower than for wild type receptor, notwithstanding the fact that 6- to 10-fold more mR139Q mutant receptors were expressed on the cell surface. In addition the EC_{50} for IP production was increased 20-fold more than expected from the effect of the mutation on GnRH binding affinity. This uncoupling is reflected in the low ε (coupling efficiency) value of 6% (Table 7).

The EC_{50} of GnRH for IP production by the mR139Q GnRH receptor (65.2 nM) was greater than the measured K_i (8.88 nM). This might appear to be inconsistent with receptor activation theory, which predicts that EC_{50} will always be less than or equal to K_i depending on the degree of receptor reserve (Loeb and Strickland, 1987; Kenakin, 1993). This discrepancy most likely results from differences in the conditions of the experiments designed to measure K_i and EC_{50}. Firstly, K_i was measured under near-equilibrium binding conditions, whereas the EC_{50} reflects IP accumulation over a time period during the early part of which GnRH binding is far from equilibrium. Secondly, K_i and EC_{50} were measured at 4°C and 37°C respectively, and a lower temperature is known to promote formation of the high agonist affinity state in some GPCRs including the GnRH receptor (Schwarz et al., 1986; this thesis **Section 3.1.4**). Both of these factors would tend to increase the measured EC_{50} relative to the K_i for any given agonist.

Previous mutational analyses of $Arg^{3.50}$ in other receptors have implicated this residue in the coupling of a number of GPCRs to their respective effector systems. Studies with the m1 muscarinic receptor reported the effects of mutating $Arg^{3.50}$ to a series of residues including Lys, Ala, Leu, Glu, Asn, and Gln (Zhu et al., 1994; Jones et al., 1995). Mutants of the m1 muscarinic receptor in which the positive charge of $Arg^{3.50}$ was replaced with neutral polar, non-polar, or negatively-charged residues showed normal antagonist and decreased agonist binding affinities, and expression levels similar to wild type. However, these mutant receptors exhibited strongly disrupted IP production (i.e. uncoupling). The charge-conserving mutation R3.50K of the m1 muscarinic receptor showed normal antagonist and increased agonist binding affinities with the normal maximal IP response being preserved, although agonist potency to stimulate IP production was decreased by 6-fold compared with wild type, indicating a moderate degree of uncoupling (Jones et al., 1995).

In rhodopsin, mutation of $Arg^{3.50}$ to Gly, Leu and Trp resulted in total uncoupling, while the R3.50K and R3.50Q mutants retained a low level of coupling (Franke et al., 1992; Min et al., 1993). In addition, reversal of the motif (ER→RE) caused impairment of coupling, while substitution of both residues with Ala (ER→AA) resulted in complete uncoupling (Franke et al., 1990, 1992). The R3.50G mutant of the gastrin-releasing peptide receptor displayed a decreased agonist binding affinity and was unable to stimulate IP production (Benya et al., 1994). The R3.50H substitution in the lutropin/choriogonadotropin receptor resulted in normal agonist binding, but decreased cAMP response (Dhanwada et al., 1996), and similar findings were reported for the R3.50H mutation in the V2 vasopressin receptor (Rosenthal et al., 1993). Mutation of the DRY motif to GGA in the AT_{1A} receptor preserved normal agonist binding affinity but abolished IP production (Ohyama et al., 1992).

In a study carried out simultaneously with work reported in this thesis, Arora et al. (1997) reported that the mR139Q mutant of the mouse GnRH receptor displayed a 50% reduction in expression at the cell surface, in addition to an impaired ability to

stimulate IP production. This is in contrast to the results reported in this thesis where an increased expression level (6- to 10-fold) at the cell surface was observed. No adequate explanation for this inconsistency can be offered at the present time. However, Arora et al. (1997) used the COS-7 cell line for the transient expression of their receptors, while the COS-1 cell line was used in the present studies. The possibility that the COS-1 and COS-7 clones could synthesize or degrade mR139Q receptors at different rates cannot not be excluded, although this seems unlikely.

Taken together, these results indicate that the positive charge of $Arg^{3.50}$ is critical for receptor-G protein coupling. All GPCR mutations resulting in a loss of this charge exhibited impaired coupling and the present findings on the GnRH receptor are consistent with this. Neither a positive charge nor a polar residue at this locus is universally required for normal receptor folding and cell surface expression. However, some GPCRs, including the GnRH receptor, show complete loss of ligand binding when a non-polar residue is substituted for $Arg^{3.50}$, indicating either gross misfolding or blocked cell surface expression. The results reported here for the GnRH receptor differ from those for the m1 muscarinic receptor and rhodopsin, in that even the conservative R3.50K substitution resulted in total abolition of agonist and antagonist binding in the GnRH receptor. Therefore, it could not be determined whether K could substitute for $Arg^{3.50}$ in coupling in the GnRH receptor. The severely impaired coupling reported in the rhodopsin R3.50K mutant (Min et al., 1993) suggests that a positive charge at this position is not the only requirement for coupling, and that $Arg^{3.50}$ participates in other hydrogen bonding or steric interactions which K cannot mimic. A clearer understanding of the role of this residue in receptor-G protein coupling will require that the effects of the R3.50K mutation be characterized in other members of the rhodopsin-like GPCRs.

The severe uncoupling of the mR139Q GnRH receptor suggests that $Arg^{3.50}$ participates in the change in receptor conformation ($R \rightarrow R^*$) which accompanies receptor activation. It raises the question whether this mutant is uncoupled because

it fails to adopt the R* conformation, or whether it can adopt an R*-like conformation (as far as the binding pocket is concerned) but nevertheless fails to activate the G protein. Since the R* conformation has a high agonist affinity (DeLean et al., 1980), the decreased agonist affinity of the mR139Q GnRH receptor observed (Figures 10 and 11) would be consistent with a failure to adopt an R* conformation. However, the mR139Q GnRH receptor also showed lower antagonist affinity than the wild type GnRH receptor (Figure 9), which would not be predicted if the decrease in agonist affinity was due to an inability to assume an R*-like conformation.

Relevant to this is the finding that, in the mouse GnRH receptor, low temperature induces a receptor state which has high agonist affinity (and therefore resembles R*, at least as far as the binding pocket is concerned), as has been previously shown in some other GPCRs (Weiland et al., 1979; Schwarz et al., 1986a, b; Lynch et al., 1988). It was therefore of interest to determine whether the mR139Q GnRH receptor showed the same temperature modulation of agonist affinity. In fact, the mR139Q GnRH receptor did show temperature modulation of agonist binding, similar to wild type GnRH receptor (Figure 15). This result indicates that while $Arg^{3.50}$ is critical for coupling, its side-chain interactions are not required for the conformational change towards a high agonist affinity state induced by low temperature.

3.2.2 **Role of Asp^{138} in coupling**

Mutagenesis studies investigating the role of the highly conserved acidic residue $Asp^{3.49}$/$Glu^{3.49}$ have produced a spectrum of effects on coupling in different receptors, ranging from uncoupling to constitutive activation. In the β_2-adrenergic and m1 muscarinic receptors the D3.49N mutation preserved high affinity agonist binding, but significantly decreased or completely abolished activation of their effector systems (Fraser et al., 1988, 1989). Similarly, the E3.49D substitution in rhodopsin resulted in decreased G_t activation (Franke et al., 1992). In contrast, constitutive G_t activation was observed in the E3.49Q mutant rhodopsin (Sakmar et al., 1989), and

the D3.49A mutation of the α_{1B}-adrenergic receptor also led to constitutive activation (Scheer et al., 1996). No effect on signal transduction was reported when $Glu^{3.49}$ was mutated to Asp or Gln in the lutropin/choriogonadotropin receptor (Wang et al., 1993). In the present study, the mD138N GnRH receptor showed no evidence of increased basal activity, but had an increased calculated coupling efficiency (ε = 310%), suggesting that this mutation perturbs the GnRH receptor conformation equilibrium slightly towards the activated state.

Arora et al. (1997) also reported decreased expression and an enhanced coupling efficiency for their mD138N mutant GnRH receptor. However, expression was decreased to 1/10th of the wild type receptor, in contrast to the 55-67% reduction reported in this thesis. As discussed above, this difference may be related to their use of COS-7 cells. In addition, their mD138N mutant GnRH receptor showed a higher rate of agonist-promoted internalization than wild type, consistent with the results presented here.

3.2.3 **Models for GPCR activation**

Because of its high degree of conservation, and its location at the membrane-cytoplasm interface, $Arg^{3.50}$ has been proposed to play a pivotal role in receptor activation (Oliveira et al., 1994; Scheer et al., 1996; Ballesteros et al., 1998).

A number of highly conserved polar residues including, $Asn^{1.50}$ of TM1, $Asp^{2.50}$ of TM2, and $Asn^{7.49}$ and $Tyr^{7.53}$ of TM7 (Asn^{53}, Asn^{87}, Asp^{318} and Tyr^{322} in the mouse GnRH receptor) (see Figure 5), are believed to form a polar pocket near the cytosol, as has been highlighted by different modelling studies (Trumpp-Kallmeyer et al., 1992; Oliveira et al., 1994, Scheer et al., 1996). The Arg-switch model of Oliveira et al. (1994) is shown in Figure 29a. This model proposes that $Arg^{3.50}$ (Arg^{139} in the mouse GnRH receptor), which is located near both the cytosol and the highly conserved polar pocket, has a 'switching' role which is expressed through two

alternative positions of its side-chain. In this model, the highly conserved $Leu^{2.46}$, four residues away from $Asp^{2.50}$, is positioned to orient the $Arg^{3.50}$ side-chain through hydrophobic interaction. When the $Arg^{3.50}$ side-chain is in the polar pocket formed by $Asn^{1.50}$, $Asp^{2.50}$, $Asn^{7.49}$ and $Tyr^{7.53}$ the switch is *off*, while the switch is *on* when the side-chain is shifted toward the cytosol where it can bind and activate the G protein (Oliveira et al., 1994).

The high degree of conservation of the acidic $Glu/Asp^{3.49}$ in GPCRs could imply that protonation of this negatively charged side-chain is part of a mechanism for receptor activation. In the model of α_{1B}-adrenergic receptor activation of Scheer et al., 1996 (Figure 29b), it was suggested that the R (inactive) state is stabilized by the network of hydrogen-bonding interactions between $Asn^{1.50}$, $Asp^{2.50}$, $Asn^{7.49}$ and $Tyr^{7.53}$ forming the polar pocket, and $Arg^{3.50}$ of the DRY sequence motif. This model of the α_{1B}-adrenergic receptor, suggests that protonation of $Glu/Asp^{3.49}$ (Asp^{138} in the mouse GnRH receptor) disrupts the salt bridge, between $Asp^{3.49}$ and Arg^{160} of the α_{1B}-adrenergic receptor on the C-terminal side of the second intracellular loop (at the bottom of TM4), which exists in the R state of the receptor. This disruption is predicted to induce helical movements which cause conformational changes in the second intracellular loop that force $Arg^{3.50}$ out of the polar pocket, thus exposing several residues to the cytosol. These residues then achieve the correct configuration for the formation of a G protein docking and binding site i.e. the R* (active) state (Scheer et al., 1996).

As discussed in **Section 1.3.3.1**, Ballesteros et al. (1998) have recently proposed the Arg-cage hypothesis, based on the GnRH receptor, as a model for GPCR activation. In particular, it is proposed that the inactive receptor conformation is stabilized by an ionic interaction between $Glu/Asp^{3.49}$ and $Arg^{3.50}$, and that the $Glu/Asp^{3.49}$ becomes protonated upon receptor activation allowing the $Arg^{3.50}$ side-chain to be released and to participate in G protein activation (Figure 29c). This

model of the GnRH receptor suggests that the conserved residues surrounding $Arg^{3.50}$ act like a cage that affects the orientation of the Arg side-chain, and that a highly conserved β-branched Ile residue ($Ile^{3.54}$) in the α-helical turn following $Arg^{3.50}$ supports the orientation of the $Arg^{3.50}$ side-chain that is required for efficient receptor-G protein interaction. The model also predicts that disruption of the cage will impair receptor function. Indeed, mutation of $Ile^{143(3.54)}$ to Ala, which eliminates the branched side-chain, resulted in considerably impaired receptor-G protein coupling, providing experimental support for this Arg-cage hypothesis (Ballesteros et al., 1998).

The present data are consistent with the above models of receptor activation for the GnRH receptor, since they predict that mutation of $Arg^{3.50}$ will disrupt receptor-G protein coupling, as has been shown here for the GnRH receptor (discussed above). Furthermore, although there is no Arg residue on the C-terminal side of the second intracellular loop of the mouse GnRH receptor, to form a salt bridge with $Asp^{138(3.49)}$, (as described above in the α_{1B}-adrenergic receptor model between Arg^{160} and $Asp^{3.49}$) there is a Lys residue ($Lys^{154(4.40)}$, two residues away from the position of Arg^{160} in the α_{1B}-adrenergic receptor) which may satisfy this requirement. Previous mutagenesis studies of the GnRH receptor are also consistent with the models of Oliveira et al. and Scheer et al. When $Asn^{87(2.50)}$ of the GnRH receptor was mutated to Asp, impaired ligand binding and signal transduction was observed (Zhou et al., 1994). Mutation of $Asp^{2.50}$ of a number of GPCRs had been shown to disrupt signal transduction (reviewed in Savarese and Fraser, 1992; Strader et al., 1994), indicating that this residue is of vital importance in the signal transduction mechanism. Moreover, mutation of $Asn^{1.50}$ of the α_{1B}-adrenergic receptor to Ala resulted in a constitutively active receptor (Scheer et al., 1996). It will be of interest to investigate the effects of mutating $Asn^{1.50}$ of the GnRH receptor, on G protein coupling and signal transduction.

Support for the role of TM7 residues in the mechanism for initially maintaining the inactive R conformation of GPCRs, followed by their isomerization from R→R* in response to agonist, has come from mutagenesis studies of the highly conserved $NP(X)_{2,3}$ Y motif, of which $Asn^{7.49}$ and $Tyr^{7.53}$ form a part. Both the substitution of $Asp^{318(7.49)}$ with Asn and replacement of $Tyr^{322(7.53)}$ with Ala markedly impaired the ability of the GnRH receptor to interact with G proteins and to couple to inositol phosphate production (Arora et al., 1996). Similar findings have been reported for the gastrin-releasing peptide (Slice et al., 1994) and AT_{1A} (Hunyady et al., 1996; Laporte et al., 1996) receptors, while mutation of $Asn^{7.49}$ to Ala resulted in complete uncoupling of the β_2-adrenergic receptor (Barak et al., 1995). These results suggest that rather than being purely an internalization motif as reviewed in **Section 1.3.3.3**, the $NP(X)_{2,3}$ Y sequence, with $Asn^{7.49}$ and $Tyr^{7.53}$ being the functional residues that interact with $Asn^{1.50}$, $Asp^{2.50}$ and $Arg^{3.50}$, may function as a critical determinant for stabilizing the R conformation of GPCRs and in mediating the changes in that conformation in response to agonist.

The present finding that the mD138N mutant receptor, in which the negative charge at position 3.49 is removed, showed an increased coupling efficiency, suggests that protonation of this residue could be involved in GnRH receptor activation. Constitutive activation of the GnRH receptor was not observed when $Asp^{138(3.49)}$ was mutated to Ala, as reported by Scheer et al. (1996) for the α_{1B}-adrenergic receptor. The observation of the present study that the mD138A mutant GnRH receptor displayed no detectable agonist binding, antagonist binding, or IP production, most likely indicates gross misfolding, or failure of expression of this receptor.

In summary, the models of Scheer et al. and Ballesteros et al. predict that protonation of $Glu/Asp^{3.49}$ results in the release of the positively charged $Arg^{3.50}$ side-chain, possibly toward the cytosol, where it can participate in G protein activation. In the models of Oliveira et al. and Scheer et al., $Arg^{3.50}$ is involved in a network of

hydrogen bonding interactions with $Asn^{1.50}$, $Asp^{2.50}$, $Asn^{7.49}$ and $Tyr^{7.53}$, while the model of Ballesteros et al. places $Arg^{3.50}$ in an ionic interaction with $Glu/Asp^{3.49}$. In all three models, these interactions are predicted to stabilize the receptor in the R state. The removal of the positive charge at position 3.50 could therefore destabilize the inactive conformation of the receptor and also disrupt receptor-G protein coupling. The model of Oliveira et al. implicates the highly conserved $Leu^{2.46}$ in modulating the orientation of the $Arg^{3.50}$ side-chain through hydrophobic interaction, while this function is fulfilled by the highly conserved $Ile^{3.54}$ in the model of Ballesteros et al. In both cases, it is a bulky β-branched side-chain (Leu/Ile) orientating the $Arg^{3.50}$ side-chain. The results of the present study are consistent with all three of the above models. Further studies are required in order to determine which model is more applicable to GnRH receptor activation.

3.2.4 **Comparison of internalization of agonist- and antagonist-occupied receptors**

Before considering the relationship of internalization to receptor activation for the GnRH receptor, it was necessary to address the related question of whether agonists and antagonists differ with respect to their internalization behaviour. A review of the literature (see **Section 1.3**) indicates that the relationship between GPCR activation and internalization, remains unclear. Some studies have reported very slow or no internalization of antagonist-occupied receptors (Hoelscher et al., 1991; Lutz et al., 1992; von Zastrow et al., 1994; Williams et al., 1998), while others have reported substantial antagonist-occupied receptor internalization (Roettger et al., 1997, Pfeiffer et al., 1998).

A simple and widely used method for measuring GPCR internalization is the acid-wash method, in which receptor-bound ligand is released by low pH, and this proved adequate for use with agonist radioligands of the GnRH receptor. However the acid-

wash method proved unsuitable for the GnRH antagonist radioligand, presumably due to strong hydrophobic interactions between the antagonist peptide and the receptor, which are not susceptible to disruption by low pH. This behaviour is not unusual for GnRH receptor antagonists, as similar observations of either very slow dissociation or a nondissociable component of antagonist binding, have previously been reported (Loumaye et al., 1984; Li et al., 1994). For this reason, the DAB cross-linking method, which has not previously been used in the GPCR field, was developed for the GnRH receptor. The DAB cross-linking method overcomes the problem previously encountered, gave similar results to the acid-wash method for agonist internalization, and thus proved suitable for use with GnRH antagonist radioligand Antagonist 26.

The results showed unequivocally that the GnRH antagonist-occupied receptors were internalized very slowly if at all, compared with agonist-occupied receptors. This result implies that receptor activation is a critical requirement for GnRH receptor internalization. However, this study should be extended to include other GnRH receptor antagonists, before this conclusion can be generalized.

3.2.5 **Role of Arg^{139} in internalization**

The present study demonstrates a role for $Arg^{3.50}$ in agonist-promoted internalization of the GnRH receptor. Internalization of the mR139Q GnRH receptor was decreased to 38-52% of wild type, measured using two independent methods. This is consistent with the reported effects of the R3.50G mutation in the gastrin-releasing peptide receptor and in the R3.50H mutation in the lutropin/choriogonadotropin receptor, both of which showed impaired agonist-promoted internalization (Benya et al., 1994; Dhanwada et al., 1996). Similar findings were reported for the mR139Q GnRH receptor in the recent work of Arora et al. (1997). Interestingly, the degree of impairment of internalization of the mR139Q GnRH receptor was less severe than the effect of the mutation on coupling, which was decreased to 6% of wild type (see

Section 3.1.1), suggesting that coupling and internalization are dissociated to some extent.

To investigate whether the decrease in internalization seen in the mR139Q GnRH receptor was due to a failure of this uncoupled receptor to activate PKC, the effect of PKC activation by phorbol ester on agonist-promoted internalization of wild type and mR139Q GnRH receptors was examined. Although PMA treatment enhanced internalization of both wild type and mR139Q GnRH receptors, it did not restore internalization of the uncoupled mutant to the wild type level. These results are consistent with those reported for mutant gastrin-releasing peptide (Benya et al., 1994) and thyrotropin-releasing hormone receptors (Nussenzveig et al., 1993b), and indicate that while PKC may play a modulatory role in agonist-promoted internalization, the impairment of internalization in the mR139Q mutant cannot be attributed solely to its inability to activate PKC.

Evidence that coupling and internalization can be dissociated has come from studies of mutant receptors that were uncoupled, but were found to internalize normally (Cheung et al., 1990; Hausdorff et al., 1990, Campbell et al., 1991). This implies that receptor-G protein coupling is not *generally* necessary for agonist-promoted internalization of all GPCRs. Conversely, impaired internalization has been reported for mutant receptors that displayed functional coupling and full activation of their effector systems (Lameh et al., 1992; Benya et al., 1993). Taken together, these studies indicate that receptor-mediated activation of a cognate G protein followed by second messenger production, is neither sufficient nor necessary for agonist-promoted internalization of GPCRs. The conclusion that emerges, is that agonist-promoted internalization is mediated by interactions of the cells internalization 'machinery' with structural motifs on the receptor which may or may not overlap with the motifs involved in G protein coupling.

Since an intact Arg-cage has been shown to be important for receptor-G protein coupling (Ballesteros et al., 1998), it was of interest to determine whether an intact Arg-cage structure is also essential for efficient internalization of the GnRH receptor.

The finding that the hI143L mutant displayed a wild type level of internalization is consistent with the results of Ballesteros et al. (1998) who reported that this mutant behaved essentially as the wild type receptor for both ligand binding and signal transduction efficiency. The branched bulky side-chain of the Leu residue is similar to that of Ile, and is evidently able to substitute for it in facilitating a favourable conformation of Arg^{139} according to the model. In contrast, when an Ala residue is placed at this position, the lack of a branched bulky side-chain would result in an incorrectly oriented Arg side-chain, based on the predictions of the Arg-cage model (Ballesteros et al., 1998), hence the inefficiency in mediating signal transduction. The present finding, that the hI143A mutant was poorly internalized, suggests that an intact Arg-cage is also essential for agonist-promoted internalization of GnRH receptor.

In summary, the findings of the present study demonstrate a critical role for $Arg^{3.50}$ in coupling of the GnRH receptor, but not in temperature modulation of agonist binding. The mR139Q GnRH receptor also showed impaired agonist-promoted internalization, which was not due to a failure to activate PKC as a result of its uncoupling. The mR139Q receptor was expressed in markedly increased numbers on the cell surface. The reasons for this are not clear, but the impaired internalization may be a contributing factor. Though highly conserved in GPCRs, the negative charge of $Asp^{3.49}$ was found to be unnecessary for coupling or internalization of the GnRH receptor. Interestingly, the mD138N mutant showed increased coupling efficiency and internalization, suggesting that the removal of the negative charge on $Asp^{138(3.49)}$ perturbs the receptor conformation equilibrium ($R \leftrightarrow R^*$) towards the activated state. In addition, the DAB cross-linking method was used for the first time for measuring the internalization of agonist- and antagonist-

occupied GPCRs. Using this method, antagonist-occupied GnRH receptors were shown not to undergo internalization, suggesting that receptor activation is a critical determinant for GnRH receptor internalization.

4 STRUCTURE-FUNCTION MAPPING OF THE CYTOPLASMIC TAIL OF THE CHICKEN GnRH RECEPTOR, IN MEDIATING AGONIST-PROMOTED INTERNALIZATION

4.1 RESULTS

As described in **Section 1.4.1**, the non-mammalian GnRH receptors possess cytoplamsic C-terminal tails, unlike their mammalian counterparts. In order to test the role of the cytoplasmic C-terminal tail in agonist-promoted internalization of the chicken GnRH receptor (Figure 6), and to map the regions required for the internalization process, several mutant chicken GnRH receptors were constructed in which either progressively larger portions of the cytoplasmic tail were deleted, or Ser and Thr residues were substituted with Ala. These receptor mutants are shown schematically in Figure 30.

4.1.1 Optimization of the acid-wash method of measuring agonist-promoted internalization of chicken GnRH receptors

The agonist ligand [His^5, D-Tyr^6]GnRH was found to give the best binding to the chicken GnRH receptor, and was used for internalization studies. In the experimental protocol used here, ^{125}I-[His^5, D-Tyr^6]GnRH binding to intact cells was allowed to reach equilibrium at 0°C, under which conditions receptor internalization does not occur. The time-course of binding of ^{125}I-[His^5, D-Tyr^6]GnRH to wild type human and chicken GnRH receptors (Figure 31) shows that binding equilibrium is approached after 5 hrs at 0°C, when 10^5 cpm ^{125}I-[His^5, D-Tyr^6]GnRH is added per well. A 5 hr pre-incubation at 0°C was therefore used for further internalization studies. Following warming of cells to 37°C, ^{125}I-[His^5, D-Tyr^6]GnRH internalization was measured as a function of time.

4.1.2 **Comparison of internalization of human and chicken GnRH receptors**

Radioligand receptor-mediated internalization, expressed as either % of total cell-associated ligand (Figure 32a) or absolute amount of internalized ligand (Figure 32b), showed markedly different kinetics for the wild type human and chicken receptors. The wild type chicken GnRH receptor showed rapid internalization (75% in 15 min), compared with much slower internalization by the wild type human receptor (7% in 15 min) (Figure 32a). The rapid internalization of the wild type chicken GnRH receptor was accompanied by a decrease in cell surface radioligand (80% in 90 min) (Figure 32c). In contrast, cell surface binding of the wild type human GnRH receptor increased slowly with time (2-fold over 90 min) (Figure 32c).

The % internalization data were fitted to equation 4 (**Section 2.9.1**) by non-linear regression, which yielded values for F_{max} and λ. The internalization rate constants (k_2) and the recycling rate constants (k_3) were derived using equations 9 and 10 (**Section 2.9.1**).

The rate constants for wild type chicken and human receptor internalization were 10.2%.min^{-1} and 0.71%.min^{-1} respectively, while the rate constants for recycling for the two receptors were similar at 2.37%.min^{-1} and 1.64%.min^{-1}. Thus the chicken receptor internalized 15-fold faster than the human receptor.

TABLE 9 AGONIST-PROMOTED INTERNALIZATION PARAMETERS FOR CHICKEN WILD TYPE AND TRUNCATED GnRH RECEPTORS

	cWT	cS366stop	cD356stop	cT351stop	cS346stop	cS337stop	hWT
✂[a]	-	10	20	25	30	39	-
F_{MAX}[b]	81.1±0.8	72.9±1.0	58.0±2.8	51.7±3.1	57.9±3.6	21.5±2.6	30.2±3.9
λ[c]	0.126±0.008	0.057±0.003	0.035±0.004	0.031±0.005	0.023±0.003	0.023±0.006	0.024±0.006
k_2[d]	10.2	4.14	2.00	1.60	1.35	0.50	0.71
k_3[e]	2.37	1.54	1.45	1.50	0.98	1.84	1.64

[a] The number of residues removed in each truncation mutant.

[b] F_{MAX} , the extrapolated maximal % internalization at equilibrium, was determined by curve fitting of mean data from three independent experiments

[c] λ, the time constant in min^{-1}, determined by curve fitting.

[d] k_2, the internalization rate constant in $\%.min^{-1}$, was calculated from equation 9, **Section 2.9.1**.

[e] k_3, the recycling rate constant in $\%.min^{-1}$, was calculated from equation 10, **Section 2.9.1**.

4.1.3 **Truncation mutations of the cytoplasmic tail of the chicken GnRH receptor**

To determine whether the cytoplasmic C-terminal tail was responsible for the rapid agonist-promoted internalization of the chicken GnRH receptor compared with its human counterpart, the tail was truncated after Ile^{336}. Maximal internalization (F_{max}) of the cS337stop mutant was decreased to 21.5±2.6% compared with 81.1±0.8% for the wild type chicken receptor (Figure 32a, Figure 33, Table 9), and was similar to that of the wild type human receptor (30.2±3.9%) (Figure 31a, Table 9). Cell surface binding of the cS337stop mutant receptor was similar to the wild type human receptor, increasing slowly with time (Figure 32c). A more truncated receptor (cR330stop) showed no detectable agonist binding and IP production (unpublished work, A. Katz and J. Lopes in our laboratory).

In order to more precisely localize the tail elements critical for the internalization process, four additional truncations were constructed and tested (Figure 33, Table 9). Maximal internalization (F_{max}) for the cS366stop, cD356stop, cT351stop, and cS346stop mutants decreased progressively with the removal of increasing numbers of tail residues (Table 9).

The parameter most dramatically affected by truncations of the tail was the internalization rate constant (k_2), which decreased by 20-fold, from 10.2 $\%.min^{-1}$ in the wild type chicken receptor, to 0.50 $\%.min^{-1}$ in the cS337stop truncation mutant (Table 9, Figure 34). In contrast, the recycling rate constant k_3 varied by a maximum of 2.4-fold over the series of truncation mutants (Table 9, Figure 34).

4.1.4 **Point-mutations of Ser and Thr residues in the cytoplasmic tail of the chicken GnRH receptor**

To identify which residues of the cytoplasmic C-terminal tail are required for agonist-promoted internalization of the chicken GnRH receptor, Ser/Thr residues were substituted with Ala. Serine and threonine residues were chosen for mutation since these amino acids have been shown previously to be sites for receptor phosphorylation, and to modulate the internalization of other of GPCRs (**Section 1.3.2.2**).

TABLE 10 AGONIST-PROMOTED INTERNALIZATION PARAMETERS FOR CHICKEN WILD TYPE AND MUTANT GnRH RECEPTORS

	cWT	cS337A	cT362S363→AA	cS366A	cT369T370→AA
F_{MAX}[a]	82.3±0.7	79.2±2.1	76.3±3.6	74.6±3.3	58.6±6.1
λ[b]	0.116±0.007	0.084±0.012	0.078±0.019	0.071±0.015	0.033±0.009
k_2[c]	9.51	6.63	5.99	5.33	1.93
k_3[d]	2.05	1.74	1.86	1.82	1.36

[a] F_{MAX} , the extrapolated maximal % internalization at equilibrium, was determined by curve fitting of mean data from three independent experiments

[b] λ, the time constant in min^{-1}, determined by curve fitting.

[c] k_2, the internalization rate constant in $\%.min^{-1}$, was calculated from equation 9, **Section 2.9.1**

[d] k_3, the recycling rate constant in $\%.min^{-1}$, was calculated from equation 10, **Section 2.9.1**

Since the truncation analysis had indicated that the largest effects on internalization were seen with the removal of the regions Ser^{337}-Ser^{346} and Asp^{356}-C-terminus (Table 9), the Ser and Thr residues in these segments were chosen for point-mutation to Ala. As shown in Figure 35 and Table 10, each of these point-mutations caused decreased internalization compared with the wild type chicken receptor, although the decreases were less marked than seen with the truncation mutants. The parameter

most affected by the Ser/Thr point-mutations was the internalization rate constant k_2. The greatest decrease in k_2 (5-fold) was seen in the $Thr^{369}Thr^{370}$ double mutant (Table 10). The recycling rate constant k_3 was much less affected by the point-mutations, showing a maximum 1.5-fold change from the wild type chicken receptor, which is probably with experimental error (Table 10).

4.1.5 **The role of dynamin in chicken GnRH receptor agonist-promoted internalization**

TABLE 11 AGONIST-PROMOTED INTERNALIZATION PARAMETERS FOR WT CHICKEN GnRHR IN THE PRESENCE OF CO-EXPRESSED WT DYNAMIN I AND MUTANT DYNAMIN I-K44A

	cWT	cWT + WT dynamin I	cWT + dynamin I-K44A
F_{MAX}[a]	77.7±1.3	75.4±1.4	71.4±1.1
λ[b]	0.16±0.02	0.15±0.02	0.081±0.005
k_2[c]	12.1	11.0	5.8
k_3[d]	3.5	3.6	2.3

[a] F_{MAX} , the extrapolated maximal % internalization at equilibrium, was determined by curve fitting of mean data from three independent experiments

[b] λ, the time constant in min^{-1}, determined by curve fitting.

[c] k_2, the internalization rate constant in $\%.min^{-1}$, was calculated from equation 9, **Section 2.9.1**

[d] k_3, the recycling rate constant in $\%.min^{-1}$, was calculated from equation 10, **Section 2.9.1**

To investigate the role of dynamin in chicken GnRH receptor internalization, the ability of wild type rat dynamin I and dominant-negative mutant dynamin I-K44A constructs to influence the internalization of co-transfected chicken GnRH receptors in COS-1 cells was tested. Agonist-promoted internalization of the wild type chicken GnRH receptor was unaffected by the presence of co-transfected dynamin I (Figure 36), whereas co-transfection of dynamin I-K44A inhibited internalization, as reflected

by the 48% decrease in the internalization rate constant k_2 (Figure 36, Table 11). The recycling rate constant k_3 was little affected by the co-expression of dynamin I-K44A, showing only a 1.5-fold change from the wild type chicken receptor in the absence of mutant dynamin I-K44A. When the amount of dominant-negative dynamin I-K44A cDNA co-transfected was increased, the level of internalization of wild type chicken GnRH receptor decreased in a dose-related manner (Figure 37). In contrast, co-transfected dynamin I-K44A had no effect on internalization of wild type human and cS337stop GnRH receptors, which lack C-terminal tails (Figure 36).

4.2 DISCUSSION

Mammalian GnRH receptors show structural features which are unique in the GPCR family, the most striking of which is the absence of a C-terminal tail (Tsutsumi et al., 1992). The recent cloning of the catfish (Tensen et al., 1997), chicken, goldfish and frog (Troskie et al., 1997) GnRH receptors has revealed that these non-mammalian receptors all possess cytoplasmic C-terminal tails. This presented the opportunity to investigate the role of the tail on GnRH receptor function. Since the C-terminal tail has been shown to be involved in internalization for a number of GPCRs (see **Section 1.3.3.4**), the effect of this structural difference on the agonist-promoted internalization of the human and chicken GnRH receptors was investigated.

For the analysis of receptor internalization data, a simple mathematical model was derived from first principles as described in **Section 2.9.1**. This model describes receptor trafficking in terms of four fluxes (r1, r2, r3, r4), and it was assumed that the rates of new receptor synthesis (r1) and receptor degradation (r4) were small compared to the rates of internalization (r2) and recycling (r3). The assumption that r4 could be neglected is reasonable on the basis of the degradation rate experimentally observed using photoaffinity-labeled GnRH receptors (**Section 5.2.2**). Furthermore, under steady-state conditions, the total number of receptors is

constant, and therefore r1 = r4 under these conditions. It is therefore not unreasonable to assume that r1 is also small compared with r2 and r3, and can be neglected in the analysis.

The experimental internalization data were fitted to the equation derived from the above model, yielding the fitted parameters F_{max} and λ. The internalization rate constant (k_2) and the recycling rate constant (k_3) were calculated from F_{max} and λ. It is important to note that decreased 'internalization' manifested by a decreased F_{max} could result either from a decrease in k_2 (internalization rate constant) **or** from an increase in k_3 (recycling rate constant). These two possibilities are depicted graphically in Figure 38. It is evident that the determination of experimental data at early time-points is required to distinguish between them.

The finding that the wild type chicken GnRH receptor displayed a markedly higher internalization rate than the wild type human receptor, suggested that the presence of the cytoplasmic tail mediated rapid chicken GnRH receptor internalization. This was confirmed by truncation of the C-terminal tail of the chicken GnRH receptor at position 337, which resulted in a receptor that behaved similarly to the wild type human receptor. Interestingly, despite their widely differing internalization rates, the chicken and human receptors displayed similar recycling rate constants.

Truncation of the cytoplasmic tail of the chicken GnRH receptor resulted in dramatic decreases in the internalization rate constant k_2 (up to 20-fold), while the recycling rate constant k_3 was little affected (less than 2.4-fold change). These data indicate that internalization specifically requires structural determinants located in the cytoplasmic tail, while recycling is relatively independent of these structural motifs. There was a progressive decrease in k_2 as more tail residues were removed, indicating that the structural determinants for internalization are located in all regions of the tail (distal to Ile^{336}). However, the relative magnitudes of the changes in the truncation mutants indicated that the major determinants of internalization lie in two

regions (a) between Asp^{356} and the C-terminus and (b) between Ser^{337} and Ile^{345}. These two regions were therefore targeted for point-mutation of Ser and Thr residues to Ala.

Each of the Ser/Thr point-mutations caused an impairment of internalization as reflected in a decreased k_2. The greatest decrease was seen in the double mutant where the doublet of Thr^{369} and Thr^{370} was mutated. In this mutant, the impairment of internalization (5-fold decrease in k_2) was equivalent to that seen in the cD356stop truncation, indicating that these residues are critical for the function of this segment, and suggesting that they may be critical sites of phosphorylation for modulation of β-arrestin binding.

Mutation of other Ser/Thr residues caused smaller decreases in k_2, suggesting that their phosphorylation may also contribute to internalization but is quantitatively less important. The recycling rate constant k_3 was affected little by any of the Ser/Thr point-mutations, including the $Thr^{369}Thr^{370}$ doublet. This confirms the conclusion from the truncation mutants, that receptor recycling is independent of structural determinants in the cytoplasmic C-terminal tail.

A shortcoming in using truncated receptors to identify signals for internalization, is that it is not possible to determine whether internalization is being regulated by specific amino acid residues or whether internalization is *also* modulated by the length of the receptor's tail. A critical length of the cytoplasmic tail, perhaps combined with a specific internalization signal, could be required to allow interaction with the internalization 'machinery' of the cell. Findings with several GPCRs, including the gastrin-releasing peptide (Benya et al., 1993), Thyrotropin-releasing hormone (Nussenzveig et al., 1993a), AT_{1A} (Hunyady et al., 1994b), Neurotensin (Chalbry et al., 1995), and δ Opioid (Trapaidze et al., 1996) receptors (**Section 1.3.3.4**), have shown that the length of the cytoplasmic tail is important, insofar as its truncation attenuated internalization rates. In contrast, truncation of the

lutropin/choriogonadotropin receptor to residue 631, which is ten amino acids distal to the conserved palmitoylation site Cys residues, caused that mutant receptor to internalize to an higher level (F_{max}) than wild type (Rodriguez et al., 1992). Interestingly, the increased internalization rate constant (k_2) observed for the 631stop truncated lutropin/choriogonadotropin receptor was coupled to an increased rate of degradation of internalized hCG hormone, presumably due to an increase in the rate at which hormone is delivered to the lysosomes. The effects of truncations of the cytoplasmic tail of β_2-adrenergic receptors are complex. Truncation of the human β_2-adrenergic receptor at position 365, which is twenty-three amino acids distal to the conserved palmitoylation site Cys residue (Cys^{341}), caused the mutant to be internalized to a higher F_{max} than wild type (Bouvier et al., 1988). Truncation of the hamster β_2-adrenergic receptor at position 354, twelve amino acids distal to the conserved palmitoylation site Cys residue (Cys^{341}), did not effect internalization (Strader et al., 1987; Cheung et al., 1989), whereas truncation at residue 345 decreased the F_{max} of internalization, the result of a decrease in the internalization rate constant (k_2) (Cheung et al., 1989).

For non-GPCRs, such as the low density lipoprotein, transferrin, human asialoglycoprotein and bovine cation-independent mannose-6-phosphate receptors, distinct small peptide sequences in the cytoplasmic tails (Asn-Pro-Val-**Tyr**, **Tyr**-Thr-Arg-Phe, **Tyr**-Gln-Asp-Leu, and **Tyr**-Lys-**Tyr**-Ser-Lys-Val respectively) all containing a tyrosine residue, have been identified as receptor internalization signals. These Tyr containing peptide sequences have been shown to occur predominantly in β-turns (Trowbridge, 1991), suggesting that a Tyr residue in the context of a β-turn is a characteristic feature of internalization signals, at least for constitutively recycling receptors. Whether or not such a motif could play a similar role in GPCRs, is unclear. However, for at least two GPCRs, the parathyroid hormone (Huang et al., 1995) and neurotensin (Chalbry et al., 1995) receptors, tyrosine has been suggested or identified to be important for internalization. Furthermore, it has also been shown

that the medium chains (μ1 and μ2) of clathrin-associated protein complexes AP-1 and AP-2 respectively (**Section 1.3.2.1**), can specifically interact with a Tyr-based signal YXXϕ (where *X* is any amino acid, and ϕ an amino acid with a bulky hydrophobic side-chain) motif (Ohno et al., 1995). The **Tyr**-Thr-Arg-**Phe** internalization signal of the transferrin receptor bears a strong resemblance to the aforementioned Tyr-based signal.

The present results may be of value in identifying an internalization motif in GPCRs. Tyrosine residues obviously do not play a critical role in GnRH receptor internalization, since the cytoplasmic tail of the chicken GnRH receptor internalizes very rapidly, yet contains no Tyr residues. The sequence Ser-Thr-Leu in the AT_{1A} receptor was determined to play an essential role in its internalization (Hunyady et al., 1994b). In addition, it was suggested that the cytoplasmic tail sequence Thr-Ser-Ile-Ser of the human B1 kinin receptor may be involved in its internalization (Faussner et al., 1998). The present results show that the Thr^{369}-Thr^{370}-Val^{371} sequence of the chicken GnRH receptor cytoplasmic tail is critical for internalization. Thus, it appears that Thr/Ser-Thr/Ser-Ile/Leu/Val may constitute a motif for internalization of some GPCRs. Interestingly, this motif is also present in the tails of several GPCRs including the β_2-adrenergic, D_1-dopamine, glucagon-like peptide-1, human interleukin-8, lutropin/choriogonadotropin, human neurokinin A, rat neurotensin, and rat substance P receptors. Its role in mediating internalization in these receptors has not been investigated and will be of interest.

Studies of GnRH receptor desensitization from this laboratory reported that the absence of a tail in mammalian GnRH receptors is correlated with an absence of rapid homologous desensitization of phospholipase C activation by these receptors (see **Section 1.4.3**). It remains to be determined whether the wild type chicken GnRH receptor undergoes rapid desensitization of its signaling function and whether or not the cytoplasmic tail plays a role in this event. The absence of the cytoplasmic

C-terminal tail in mammalian GnRH receptors and its presence in non-mammalian vertebrate GnRH receptors suggests this may have physiological correlates in mammals and non-mammals. The absence of the cytoplasmic tail may have been selected to prevent rapid desensitization and internalization of mammalian GnRH receptors, in order to keep a large number of active receptors on the cell surface to allow a protracted LH surge over several hours which is required for ovulation in mammals. In contrast the chicken has a one day cycle, which includes a substantially shorter LH surge of no more than 2 hrs (Furr et al., 1973).

Since Dynamin (**Section 1.3.2.3**) has been shown to modulate the internalization of GPCRs, it was of interest to investigate its effect on chicken GnRH receptor internalization. Dynamin colocalizes with clathrin (has been demonstrated to bind to a component of AP-2, **Section 1.3.2.3**) and contributes to the early stages of internalization by catalyzing the GTP-dependent disconnection of CCVs from the plasma membrane. The finding that overexpression of dominant-negative dynamin I-K44A mutant led to a decrease in wild type chicken GnRH receptor internalization implies that internalization proceeds via a dynamin-dependent pathway, and thus could internalize via clathrin-coated vesicles. That overexpression of wild type dynamin I did not significantly increase chicken GnRH receptor internalization could be explained by the possibility that COS-1 cells (used here) have an endogenous over-abundance of dynamin, and that only overexpression of a dominant-negative mutant dynamin that competes with wild type dynamin, will show any effect.

In conclusion, the findings of the present study show that the cytoplasmic C-terminal tail of the chicken GnRH receptor is essential for agonist-promoted internalization. The internalization rate of the wild type chicken GnRH receptor internalization was 15-fold more rapid than its human counterpart, and truncation of the cytoplasmic tail resulted in a decrease in internalization rate to a level equivalent to the wild type human receptor. These results suggest that the loss of the tail during evolution of the mammalian GnRH receptor may be related to its effects on internalization. In

contrast, the recycling rates of human, chicken, and truncated chicken receptors were found to be similar. The results show that a Thr-Thr doublet located near the C-terminal end are the most important residues. Comparison of the present data with those obtained for other GPCRs shows that the motif Thr/Ser-Thr/Ser-Ile/Leu/Val may be an internalization sequence. The present results also provide evidence that internalization of the chicken GnRH receptor is dependent on the GTPase dynamin, thus providing evidence that this process proceeds via clathrin-coated vesicles.

5 PHOTOAFFINITY LABELING STUDIES OF THE MOUSE GnRH RECEPTOR

5.1 RESULTS

A quantitative description of receptor trafficking requires that an estimate of receptor degradation rate be determined. Photoaffinity labeled receptors can be visualized on SDS-PAGE gels using autoradiography, allowing receptor degradation to be visualized by a decrease in molecular weight and/or a decrease in labeling intensity. A prerequisite for such studies is that the photoaffinity ligand should cross-link to the receptor in a relatively stable manner (i.e. the rate of decay of the cross-link should be slow compared with the receptor degradation rate).

5.1.1 Determination of the *in vitro* stability of the ^{125}I-[*N*-azidobenzoyl-D-Lys^{6}]GnRH cross-link to the GnRH receptor

UV-induced cross-linking of ^{125}I-[*N*-azidobenzoyl-D-Lys^{6}]GnRH to mouse GnRH receptors endogenously expressed in the mouse pituitary gonadotrope αT3-1 cell-line, followed by SDS-PAGE and autoradiography revealed specific labeling of broad bands with apparent molecular weights of ±45 kDa (Figure 39, lanes 1 and 2). The broadness of the band was due to heterogeneity of receptor glycosylation, as deglycosylation of the labeled membranes with peptide N-glycosidase F (PN-gase F) resulted in visualization of a sharp band of 32 kDa (Figure 39, lanes 3 and 4). The sharp bands appearing at ±27kDa (Figure 39, lanes 1 and 2) are probably non-specifically labeled membrane components, or they may represent degraded GnRH receptor fragments. The absence of bands of lower molecular weight than the deglycosylated receptor (Figure 39, lanes 3 and 4) suggests the absence of receptor degradation during the 18 hr deglycosylation step.

To assess the *in vitro* stability of the covalent cross-link of ^{125}I-[*N*-azidobenzoyl-D-Lys^{6}]GnRH to the GnRH receptor, photoaffinity labeled membranes were incubated at 37°C for various time-points up to 24 hrs. To prevent proteolytic degradation of the ligand-receptor complex, various protease inhibitors were included during the incubation period. In Figure 40, receptor degradation (i.e. the appearance of lower molecular weight fragments) is already evident at 0 hrs incubation in the absence of protease inhibitors (leupeptin and PMSF), while inclusion of the inhibitors prevented GnRH receptor degradation up to approximately 6 hrs, after which degradation was clearly visible (Figure 40, lanes 9-12). In the experiment shown in Figure 42, EDTA was also included as a protease inhibitor, in addition to leupeptin and PMSF. Once again, the protease inhibitors were effective at preventing receptor degradation up to approximately 6 hrs. Since there was no degradation seen in Figure 39, this would indicate that there was variability of degradation in the different receptor preparations.

Pre-incubation of labeled membranes for 10 min at 60°C was performed to heat inactivate proteases (Figure 43), demonstrating a slow decrease in labeling over 24 hours. Receptor degradation (i.e. the appearance of lower molecular weight fragments) was evident in lanes 7 and 8 (Figure 43) in which no 60°C inactivation step was included.

The data of Figure 40 were quantitated and fitted to the exponential decay equation, as shown in Figure 41. This yielded a value of 0.05%.min^{-1} for the *in vitro* decay rate constant, corresponding to a half-life of 23.2 hrs.

5.1.2 **Intracellular degradation of ^{125}I-[*N*-azidobenzoyl-D-Lys^6]GnRH cross-linked GnRH receptor**

Intact cells were photoaffinity labeled with ^{125}I-[*N*-azidobenzoyl-D-Lys^6]GnRH to determine the rate of degradation of the ligand-receptor complex *in vivo*. Intact αT3-1 cells, covalently labeled with ^{125}I-[*N*-azidobenzoyl-D-Lys^6]GnRH were returned to cell culture and incubated at 37°C for various time-points up to 24 hrs. The cells were then harvested and processed for SDS-PAGE and autoradiography as described in **Section 2.8.3**. The radioactivity in the GnRH receptor bands (Figure 44) were quantitated and the data fitted by non-linear regression to a single component exponential equation (Figure 45), yielding a half-life of degradation of 4.8 hrs, which corresponds to a degradation rate constant k_4 = 0.24%.min^{-1}. Radioactivity released into the medium at each time-point, mirrored the degradation of labeled GnRH receptors (Figure 45), while the total radioactivity at each time-point remained constant.

5.2 **DISCUSSION**

The results of the present study demonstrate that the cross-link between the mouse GnRH receptor and the photoreactive GnRH analog, ^{125}I-[*N*-azidobenzoyl-D-Lys^6]GnRH is stable enough to allow an estimate of the rate of intracellular degradation of the mouse GnRH receptor, which was shown to have a half-life of 4.8 hrs.

The importance of determining the stability of the cross-link is clear. Should the photoreactive agonist begin to dissociate from its binding pocket on the receptor as a result of an unstable cross-link, the decreasing intensity of the bands in Figure 44 would be due to a combination of both degradation and photoreactive agonist dissociation. It was necessary to include protease inhibitors during the incubation

steps, since rapid receptor degradation was observed in some experiments (Figures 40 and 42). The choice of inhibitors, leupeptin, PMSF, and EDTA, was made to cover as broad a spectrum of proteases as possible. Nevertheless, these protease inhibitors were ineffective after approximately 6 hrs (Figures 40 and 42). An alternative means of inactivating proteases is a high temperature pre-incubation step (60°C for 10 min). However, this procedure had the undesirable effect of causing aggregation of photoaffinity-labeled GnRH receptors, rendering their quantitation in gels unreliable.

The slow decrease in labeling intensity with incubation *in vitro* could be due to (a) chemical instability of the cross-link, or (b) proteolytic degradation by proteases resistant to the inhibitors used. The latter interpretation is the most likely, as residual proteolytic degradation in the presence of inhibitors was revealed by a decrease in molecular size of the labeled bands at late time points. However, a combination of proteolytic degradation plus cross-link instability cannot be ruled out by the present data. To estimate the true *in vivo* degradation rate, the decrease in receptor labeling observed *in vivo* over time should be corrected for the contribution due to chemical decay of the cross-link. In quantitative terms, the intensity I(t) of the labeled band would decrease with time, t, according to the equation

$$I(t) = I_0 \cdot e^{-k5.t}$$

or,

$$I(t) = I_0 \cdot e^{-(k4 + k5)t}$$

where k_4 is the receptor degradation rate constant (see **Section 2.9.1**) and k_5 is the cross-link decay rate constant. Thus to correct for cross-link decay, the estimated value for k_5 should be subtracted from the observed *in vivo* decay rate constant.

As the contribution of decay due to protease activity could not be accurately quantitated, the present data show only that the cross-link used in these experiments is either stable or decays slowly, with a maximum estimate of $k_5 = 0.05\%.min^{-1}$.

If the cross-link is assumed to be stable, the *in vivo* degradation rate constant k_4 is estimated to be 0.24%.min^{-1}. However, if this assumption were incorrect, and the correction for cross-link decay is applied, a value of k_4 = 0.19%.min^{-1} is obtained. In any event, the cross-link is sufficiently stable not to influence the overall interpretation of the results.

The methodology of the protocols presented in this chapter were initially designed in order to monitor the intracellular trafficking of the photoaffinity-labeled GnRH receptor on SDS-polyacrylamide gels. The advantage of using a photoaffinity-labeled receptor, is that the progress of the receptor can be followed. The widely used acid-wash method for measuring internalization, in reality measures *ligand* rather than receptor internalization. Thus, once the ligand had dissociated from the receptor, assuming it does, the pathway that the receptor follows cannot be further characterized. Utilizing a photoaffinity ligand, which is covalently attached to the receptor, an entire 'round-trip' of endocytosis including, internalization, recycling, and degradation could, in theory, be characterized.

In order to monitor the intracellular pathway of a photoaffinity-labeled receptor, those receptors on the cell surface must be distinguished from intracellular receptors. Since the covalently attached photoaffinity ligand cannot be removed by a low pH acid-wash, an alternative means would be to modify cell surface receptors. Protease digestion of cell surface receptors in intact cells was tested. Those receptors on the cell surface at the time of protease treatment would be digested, and would run at a lower molecular weight on the SDS-polyacrylamide gel. The intracellular receptors would be protected from protease digestion and would run at the normal expected molecular weight. By quantitating the radioactivity in the two species of bands in each lane, the relative fractions of internal and cell surface receptors could then be determined.

The presence of two sites for trypsin cleavage (Lys residues) in the extracellular N-terminal domain of the GnRH receptor suggested that trypsin could be utilized. Trypsin digestion of cell surface-labeled GnRH receptors was tested under varying conditions, but failed to produce digested cell surface receptors (data not shown). This could have been due to the inability of the enzyme to access the cleavage site due to the lysine residue being either too close or embedded in, the plasma membrane.

The simple mathematical model derived in **Section 2.9.1**, and used for the analysis of receptor internalization data, describes receptor trafficking in terms of four fluxes (r1, r2, r3, r4). One of the assumptions made in this derivation was that receptor degradation (r4) was small compared to rates of internalization (r2) and recycling (r3). The observed *in vivo* half-life of degradation of the mouse GnRH receptor was 4.8 hrs corresponding to a rate constant (k_4) of 0.24%.min^{-1}. As discussed above, this is a maximum estimate, and k_4 could be lower if corrected for cross-link decay. Since the rate constant for degradation (k_4) is an order of magnitude smaller than the recycling rate constant (k_3), it is justifiable to omit k_4 from the model.

The results show that an internalized GnRH receptor has a much higher probability of being recycled than degraded. In most experiments in this thesis, agonist-promoted internalization of the GnRH receptor was measured over a period of only 90 min, during which time receptor degradation would be of the order of 20% and will therefore not significantly affect interpretation of the results. Furthermore, if the loss of covalent cross-link between ligand and receptor is taken into account, then the rate of degradation of GnRH receptors would in fact be even slower than that observed in Figure 44. It is evident from these results that GnRH receptors covalently labeled with tethered GnRH agonist must, on average, undergo several rounds of internalization and recycling, before being degraded, in the course of an experiment such as that shown in Figure 44.

6 REFERENCES

Anderson, L., McGregor, A., Cook, J.V., Chilvers, E. and Eidne, K.A. (1995) Rapid desensitization of GnRH-stimulated intracellular signaling events in αT3-1 and HEK-293 cells expressing the GnRH receptor. Endocrinol. 136, 5228-5232.

Antonelli, M., Olate, J., Allende, C. and Allende, J. (1991) Polylysine activates membrane-bound adenylyl cyclase from Xenopus laevis oocytes through the G_s transducing protein. Comp. Biochem. Physiol. B 99, 827-852.

Arden, J.R., Nagata, O., Shockley, M.S., Philip, M., Lameh, J. and Sadee, W. (1992) Mutational analysis of the third cytoplasmic loop domains in G protein coupled of the Hm1 muscarinic receptor. Biochem. Biophys. Res. Comm. 188, 1111-1115.

Arora, K.K., Sakai, A. and Catt, K.J. (1995) Effects of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor. J. Biol. Chem. 270, 22820-22826.

Arora, K.K., Cheng, Z. and Catt, K.J. (1996) Dependence of agonist activation on an aromatic moiety in the DPLIY motif of the gonadotropin-releasing hormone receptor. Mol. Endocrinol. 10, 979-986.

Arora, K.K., Cheng, Z. and Catt, J.K. (1997) Mutations of the conserved DRS motif in the second intracellular loop of the gonadotropin-releasing hormone receptor affects expression, activation, and internalization. Mol. Endocrinol. 11, 1203-1212.

Ashworth, R., Yu, R., Nelson, E.J., Dermer, S., Gershengorn, M.C. and Hinkle, P.M. (1995) Visualization of the thyrotropin-releasing hormone receptor and its ligand during endocytosis and recycling. Proc. Natl. Acad. Sci. USA. 92, 512-516.

Ballesteros, J.A. and Weinstein, H. (1996) Integrated methods for the construction of three-dimensional models and computational probing of structure-function relations in G protein-coupled receptors. Methods. Neurosci. 25, 366-430.

Ballesteros, J.A., Kitanovic, S., Guarnieri, F., Davies, P., Fromme, B.J., Konvicka, K., Chi, L., Millar, R.P., Davidson, J.S., Weinstein, H. and Sealfon S.C. (1998) Functional microdomains in G protein-coupled receptors: The conserved arginine cage motif in the gonadotropin-releasing hormone receptor. J. Biol. Chem. 273, 10445-10453.

Balmforth, A.J., Lee, A.J., Bajaj, B.P.S., Dickinson, C.J., Warburton, P. and Ball, S.G. (1995) Functional domains of the C-terminus of the rat angiotensin AT_{1A} receptor. Eur. J. Pharmacol. 291, 135-141.

Barak, L.S., Tiberi, M., Freedman, N.J., Kwatra, M.M., Lefkowitz, R.J. and Caron, M.G. (1994) A highly conserved tyrosine residue in G protein coupled receptors is required for agonist-mediated β_2-adrenergic receptor sequestration. J. Biol. Chem. 269, 2790-2795.

Benya, R.V, Fathi, Z., Battey, J.F. and Jensen, R.T. (1993) Serines and threonines in the gastrin-releasing peptide receptor carboxy terminus mediate internalization. J. Biol. Chem. 268, 20285-20290.

Benya, R.V., Akeson, M., Mrozinski, J., Jensen, R.T. and Battey, J.F. (1994) Internalization of the gastrin-releasing peptide receptor is mediated by both phospholipase C-dependent and -independent processes. Mol. Pharmacol. 46, 495-501.

Boge, M., Wyss, S., Bonifacino, J.S. and Thali, M. (1998) A membrane-proximal tyrosine-based signal mediates internalization of the HIV-1 envelope glycoprotein via interaction with AP-2 clathrin adaptor. J. Biol. Chem. 273, 15773-15778.

Bouvier, M., Leeb-Lundberg, L.M.F., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. (1986) Regulation of adrenergic receptor function by phosphorylation. Effects of agonist occupancy on phosphorylation of α_1- and β-adrenergic receptors by protein kinase C and the cyclic AMP-dependent kinase. J. Biol. Chem. 262, 3106-

Bouvier, M., Hausdorff, W.P., DeBalsi, A., O'Dowd, B.F., Kobilka, B.K., Caron, M.G. and Lefkowitz, R.J. (1988) Removal of phosphorylation sites from the β-adrenergic receptor delays onset of agonist-promoted desensitization. Nature (Lond.) 333, 370-373.

Bucci, C., Parton, R.G., Mather, I.H., Stunnenberg, H., Simons, K., Hoflack, B. and Zerial, M. (1992) The small GTPase Rab5 functions as a regulatory factor in the early endocytic pathway. Cell 70, 715-728.

Campbell, P.T., Hnatowich, M., O'Dowd, B.F., Caron, M.G., Lefkowitz, R.J. and Hausdorff, W.P. (1991) Mutations of the human β_2-adrenergic receptor that impair coupling to G_s interfere with receptor down-regulation but not sequestration. Mol. Pharmacol. 39, 192-198.

Caron, M.G. and Lefkowitz, R.J. (1993) Catecholamine receptors: structure, function, and regulation. Rec. Prog Horm. Res. 48, 277-290.

Cassina, M.P., Grantham, K.D. and Neill, J.D. (1997) A temporally intermediate mode of gonadotropin-releasing hormone-induced desensitization of luteinizing hormone secretion. Mol. Cell. Endocrinol. 132, 53-59.

Chaki, S., Deng-Fu, G., Yoshiaki, Y., Ohyama, K., Tani, M., Mizukoshi, M., Shirai, H. and Inagami, T. (1994) Role of carboxyl tail of the rat angiotensin II type 1A receptor in agonist-induced internalization of the receptor. Kidney. Int. 46, 1492-1495.

Chalbry, J., Botto, J-M., Nouel, D., Beaudet, A., Vincent, J-P. and Mazella, J. (1995) Thr-422 and Thr-424 residues in the carboxyl terminus are critical for the internalization of the rat neurotensin receptor. J. Biol. Chem. 270, 2439-2442.

Cheung, A.H., Sigal, I.S., Dixon, R.A. and Strader, C.D. (1989) Agonist-promoted sequestration of the β_2-adrenergic receptor requires regions involved in functional coupling with Gs. Mol. Pharmacol. 35, 132-138.

Cheung, A.H., Dixon, R.A., Hill, W.S., Sigal, I.S. and Strader, C.D. (1990) Separation of the structural requirements for agonist-promoted activation and sequestration of the β-adrenergic receptor. Mol. Pharmacol. 37, 775-779.

Chi, L., Zhou, W., Prikhozhan, A., Flanagan, C., Davidson, J.S., Golembo, M., Illing, N., Millar, R.P. and Sealfon, S.C. (1993) Cloning and characterization of the human GnRH receptor. Mol. Cell. Endocrinol. 91, R1-6.

Childs, G.V., Naor, Z., Hazum, E., Tibolt, R., Westlund, K.N. and Handcock, M.B. (1983) Localization of biotinylated gonadotropin-releasing hormone with avidin-biotin peroxidase complexes. J. Histochem. Cytochem. 21, 1422

Childs, G.V., Hazum, E., Amsterdam, A., Limor, R. and Noar, Z. (1986) Cytochemical evidence for different routes of gonadotropin-releasing hormone processing by large gonadotropes and granulosa cells. Endocrinology 119, 1329-1338.

Chu, P., Murray, S., Lissin, D. and von Zastrow, M. (1997) Delta and kappa opioid receptors are differentially regulated by dynamin-dependent endocytosis when activated by the same alkaloid agonist. J. Biol. Chem. 272, 27124-27130.

Ciechanover, A. (1994) The ubiquitin-proteosome proteolytic pathway. Cell 79, 13-21.

Conchon S., Peltier N., Corvol, P. and Clauser, E. (1998) A noninternalized nondesensitized AT_{1A} receptor transduces an amplified ANG II signal. Am. J. Physiol. 274, E336-E345.

Conn, P.M. and Hazum, E. (1981) Luteinizing hormone release and gonadotropin-releasing hormone (GnRH) receptor internalization: independent actions of GnRH. Endocrinology 109, 2040-2045.

Conn, P.M., Smith, R.G. and Rogers, D.C. (1981) Stimulation of pituitary gonadotropin release does not require internalization of ghonadotropin-releasing hormone. J. Biol. Chem 256, 1098-1110.

Daaka, Y., Luttrell, L.M., Della Rocca, G.J., Ferguson, S.S.G., Caron, M.G. and Lefkowitz, R.J. (1998) Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. J. Biol. Chem. 273, 685-688.

Davidson, J.S., Wakefield, I.K., Sohnius, U., van der Merwe, P.A. and Millar, R.P. (1990) A novel extracellular nucleotide receptor coupled to phosphoinositidase-C in pituitary cells. Endocrinology 126, 80-87.

Davidson, J.S., van der Merwe, PA., Wakefield, I., Millar, RP. (1991) Mechanisms of luteinizing hormone secretion: new insights from studies with permeabilized cells. Mol. Cell. Endocrinol. 76, C33-38.

Davidson, J.S., Wakefield, I.K. and Millar, R.P. (1994) Absence of rapid desensitization of the mouse gonadotropin-releasing hormone receptor. Biochem. J. 300, 299-302.

Davidson, J.S., Flanagan, C.A., Zhou, W., Becker, I., Elario, R., Emeran, W., Sealfon, S.C. and Millar, R.P. (1995) Identification of N-glycosylation sites in the gonadotropin-releasing hormone receptor: Role in receptor expression and not binding. Mol. Cell. Endocrinol. 107, 241-245

Davidson, J.S., Flanagan, C.A., Davies, P.D., Myburgh, D., Elario, R., Forrest-Owen, W., and McArdle, C.A. (1996) Incorporation of an addtional glycosylation site enhances expression of functional human gonadotropin-releasing hormone receptor. Endocrinology, 4, 207-212.

Davidson, J.S., Assefa, D., Pawson, A.J., Davies, P., Hapgood, J., Becker, I., Flanagan, C., Roeske, R and Millar, R.P. (1997) Irreversible activation of the gonadotropin-releasing hormone receptor by photoaffinity cross-linking: localization of attachment site to Cys residue in N-terminal segment. Biochemistry 36, 12881-12889.

Debburman, S.K., Kunapuli, P., Benovic, J.L. and Hosey, M.M. (1995) Agonist-dependent phosphorylation of human muscarinic receptors in Spodoptera frugiperda insect cell membranes by G protein-coupled receptor kinases. Mol Pharmacol. 47, 224-233.

DeLean, A., Stadel, J.M. and Lefkowitz, R.J. (1980) A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled β-adrenergic receptor. J. Biol. Chem. 255, 7108-7117.

Diviani, D., Lattion, A-L., Larbi, N., Kunapuli, P. and Pronin, A. (1996) Effect of different G protein-coupled receptor kinases on phosphorylation and desensitization of the α_{1B}-adrenergic receptor. J. Biol. Chem. 271, 5049-5058.

Dhanwada, K.R., Vijapurkar, U. and Ascoli, M. (1996) Two mutations of the Lutropin/choriogonadotropin receptor that impair signal transduction also interfere with receptor-mediated endocytosis. Mol. Endocrinol. 10, 544-554.

Duello, T.M., Nett, T.M. and Farquhar, M.G. (1983) Fate of gonadotropin-releasing hormone agonist internalized by rat pituitary gonadotrophs. Endocrinology 112, 1-10.

Dupree, P., Parton, R.G., Raposo,, G., Kurzchalia, T.V. and Simons, K. (1993) Caveolae and sorting in the *trans*-Golgi network of epithelial cells. EMBO J. 12, 1597-1605.

Eason, M.G., Moreira, S.P. and Liggett, S.B. (1995) Four consecutive serines in the third intracellular loop are sites for β-adrenergic receptor kinase-mediated phosphorylation of the α_{2A}-adrenergic receptor. J. Biol. Chem. 270, 4681-4688.

Faussner, A., Proud, D., Towns, M. and Bathon, J.M. (1998) Influence of the carboxy termini of human B1 and B2 kinin receptors on receptor sequestration, ligand internalization, and signal transduction. J. Biol. Chem. 273, 2617-2623.

Ferguson, S.S.G., Ménard, L., Barak, L.S., Koch, W.J., Colapietro, A-M. and Caron, M.G. (1995) Role of phosphorylation in agonist-promoted β_2-adrenergic receptor sequestration: rescue of a sequestration-defective mutant receptor by βARK1. J. Biol. Chem. 270, 24782-24789.

Ferguson, S.S.G., Downey, III W.E., Colapietro, A-M., Barak, L.S., Ménard, L. and Caron, M.G. (1996a) Role of β-Arrestin in mediating agonist-promoted G protein-coupled receptor internalization. Science 271, 363-366.

Ferguson, S.S.G., Zhang, J., Barak, L.S. and Caron, M.G. (1996b) Signal termination and compartmentalization. 4th IUBMB conference, Edinburgh 24, 953-958.

Flanagan, C.A., Fromme, B.J., Davidson, J.S. and Millar, R.P. (1998) A high affinity gonadotropin-releasing hormone tracer, radio-iodinated at position six,

facilitates analysis of mutant gonadotropin-releasing hormoone receptors. Endocrinollogy, in press.

Fonseca, M.I., Button, D.C. and Brown, R.D. (1995) Agonist regulation of α_{1B}-adrenergic receptor subcellular distrubution and function. J. Biol. Chem. 270, 8902-8909.

Fra, M., Williamson, E., Simons, K. and Parton, R.G. (1994) Detergent-insoluble glycolipid microdomains in lymphocytes in the absence of caveolae. J. Biol. Chem. 269: 30745-30748.

Franke, R.R., König, B., Sakmar, T.P., Khorana, H.G. and Hofmann, K.P. (1990) Rhodopsin mutants that bind but fail to activate transducin. Science 250, 123-125.

Franke, R.R., Sakmar, T.P., Graham, R.M. and Khorana, H.G. (1992) Structure and function in rhodopsin. Studies of the interaction between the rhodopsin cytoplasmic domain and transducin. J. Biol. Chem. 267, 14767-14774.

Fraser, C.M., Chung, F.Z., Wang, C.D. and Venter, J.C. (1988) Site-directed mutagenesis of human beta-adrenergic receptors: substitution of aspartic acid-130 by asparagine produces a receptor with high-affinity agonist binding that is uncoupled from adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. 85, 5478-5482.

Fraser, C.M., Wang, C.D., Robinson, D.A., Gocayne, J.D. and Venter, J.G. (1989) Site-directed mutagenesis of m1 Muscarinic acetylcholine receptors: Conserved aspartic acids play important roles in receptor function. Mol. Pharmacol. 36, 840-847.

Fredericks, Z.L., Pitcher, J.A. and Lefkowitz, R.J. (1996) Identification of the G protein-coupled receptor kinase phosphorylation sites in the human beta2-adrenergic receptor. J. Biol. Chem. 271,13796-13803.

Freedman, N.J., Liggett, S.B., Drachman, D.E., Pei, G., Caron, M.G. and Lefkowitz, R.J. (1995) Phosphorylation and desensitization of the human β_1-adrenergic receptor: involvement of G protein-coupled receptor kinases and cAMP-dependent protein kinase. J. Biol. Chem. 270, 17953-17961.

Fukushima, Y., Asano, T., Takata, K., Funaki, M., Ogihara, T., Anai, M., Tsukuda, K., Saitoh, T., Katagiri, H., Aihara, M., Matsuhashi, N., Oka, Y. and Sugano, K. (1997) Role of the C terminus in Histamine H2 receptor signaling, desensitization, and agonist-indiced internalization. J. Biol. Chem. 272, 19464-19470.

Furr, B.J.A., Bonney, R.C., England, R.J. and Cunningham, F.J. (1973) Luteinizing hormone and progesterone in peripheral blood during the ovulatory cycle of the hen *Gallus domesticus*. J. Endocrinol. 57, 159-169.

Gabilondo, A.M., Hegler, J., Boivan-Jahns, V., Hein, L. and Lohse, M.J. (1997) A dileucine motif in the C terminus of the β_2-adrenergic receptor is involved in receptor internalization. Proc. Natl. Acad. Sci. USA 94, 12285-12290.

Gagnon, A.W., Kallal, L. and Benovic, J.L. (1998) Role of clathrin-mediated endocytosis in agonist-induced down-regulation of the β_2-adrenergic receptor. J. Biol. Chem. 273, 6976-6981.

Garland, A.M., Grady, E.F., Lovett, M., Vigna, S.R., Frucht, M.M., Krause, J.E. and Bunnett, N.W. (1996) Mechanisms of desensitization and resensitization of G protein-coupled neurokinin$_1$ and neurokinin$_2$ receptors. Mol. Pharmacol. 49, 438-446.

Glickman, J.N., Conibear, E. and Pearse, B.M.F. (1989) Specificity of binding of clathrin adaptors to signals on the mannose-6-phosphate/insulin-like growth factor II receptor. EMBO J. 8, 1041-1047.

Go, W.Y., Holicky, E.L., Hadac, E.M., Rao, R.V. and Miller, L.J. (1998) Identification of a domain in the carboxy terminus of CCK receptor that affects its intracellular trafficking. Am. J. Physiol. 275, G56-G62.

Goldberg, A.L. and Rock, K.L. (1992) Proteolysis, proteosomes and antigen presentation. Nature 357, 375-379.

Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russell, D.W. and Schneider, W.J. (1985) Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. Annu. Rev. Cell Biol. 1, 1-39.

Goodman, Jr., O.B., Krupnick, J.G., Santini, F., Gurevich, V.V., Penn, R,B., Gagnon, A.W., Keen J.H. and Benovic, J.L. (1996) β-arrestin acts as a clathrin adaptor in endocytosis of the β_2-adrenergic receptor. Nature 383, 447-450.

Goodman, Jr., O.B., Krupnick, J.G., Gurevich, V.V., Benovic, J.L. and Keen, J.H. (1997) Arrestin/clathrin interaction: Localization of the arrestin binding locus to the clathrin terminal domain. J. Biol. Chem. 272, 15017-15022.

Grady, E.F., Garland, A.J., Gamp, P.D., Lovett, M., Payan, D.G. and Bunnett, N.W. (1995a) Delineation of the endocytic pathway of substance P and the seven transmembrane domain NK1 receptor. Mol. Biol. Cell. 6, 509-524.

Grady, E.F., Slice L.W., Brant, W.O., Walsh, J.H., Payan, D.G. and Bunnett, N.W. (1995b) Direct observation of the endocytosis of gastrin-releasing peptide and its receptor. J. Biol. Chem. 270, 4603-4611.

Gurevich, V.V., Dion, S.B., Onorato, J.J., Ptasienski, J., Kim, C.M., Sterne-Marr, R., Hosey, M.M. and Benovic, J.L. (1995) Arrestin interactions with G protein-coupled receptors: direct binding studies of wild type and mutant arrestins with rhodopsin, β_2-adrenergic, and m2 muscarinic cholinergic receptors. J. Biol. Chem. 270, 720-731.

Haga, K.K., Kameyama, K., Haga, T., Kikkawa, U., Shiozaki, K. and Uchiyama, H. (1996) Phosphorylation of human m1 muscarinic acetylcholine receptors by G protein-coupled receptor kinase 2 and protein kinase C. J. Biol. Chem. 271,2776-2782.

Hall, A. (1990) The cellular functions of small GTP-binding proteins. Science 249, 635-640.

Hall, A. (1993) Ras-related proteins. Curr Opin Cell Biol 1993, 265-268

Hausdorff, W.P., Bouvier, M., O'Dowd, B.F., Irons, G.P., Caron, M.G. and Lefkowitz, R.J. (1989) Phosphorylation sites on two domains of the β_2-adrenergic receptor are involved in distict pathways of receptor desensitization. J. Biol. Chem. 264, 12657-12665.

Hausdorff, W.P., Hnatowich, M.G., O'Dowd, B.F., Caron, M.G. and Lefkowitz, R.J. (1990) A mutation of the β_2-adrenergic receptor impairs activation of adenylyl cycalse without affecting high affinity agonist binding. J. Biol. Chem. 265, 1388-1393.

Hausdorff, W.P., Campbell, P.T., Ostrowski, J., Yu, S.S., Caron, M.G. and Lefkowitz, R.J. (1991) A small region of the β-adrenergic receptor is selectively involved in its rapid regulation. Proc. Natl. Acad. Sci. USA 88, 2979-2983.

Hazum, E., Cuatrecases, P., Marian, J. and Conn, P.M. (1980) Receptor-mediated internalization of fluorescent gonadotropin-releasing hormone by pituitary gonadotropes. Proc. Natl. Acad. Sci, USA. 77, 6692-6695.

Hazum, E. (1981a) Photaffinity inactivation of gonadotropin-releasing hormone receptors. FEBS letters 128, 111-113.

Hazum, E. (1981b) Photoaffinity labeling of luteinizing hormone releasing hormone receptor of rat pituitary membrane preparations. Endocrinology 109, 1281-1283

Hazum E. and Nimrod, A. (1982a) Photoaffinity labeling and fluoroscent distrubution studies of gonadotropin-releasing hormone receptors in ovarian granulosa cells. Proc. Natl. Acad. Sci. USA 79, 1747.

Hazum, E., Meidan, R., Keinan, D., Okon, E., Koch, Y., Lindner, H.R. and Amsterdam, A. (1982b) A novel method for localization of gonadotropin-releasing hormone receptors. Endocrinology 111, 2135-2137.

Hazum, E., Meidan, R., Liscovitch, M., Keinan, D., Linder, H.R. and Koch, Y. (1983a) Receptor-mediated internalization of LHRH antagonists by pituitary cells. Mol. Cell. Endocrinol. 30, 291-301.

Hazum, E. (1983b) Photoaffinity labeling of peptide hormone receptors. Endocr. Rev. 4, 352-362.

Hazum, E. and Keinan, D. (1984) Testicular GnRH receptors: photoaffinity labeling and fluorescence distribution studies. Peptides 5, 119-123.

Hazum, E., Koch, Y., Liscovitch, M. and Amsterdam, A. (1985) Intracellular pathways of receptor-bound GnRH agonist in pituitary gonadotropes. Cell. Tissue Res. 239, 3-8.

Hermans, E., Octave, J.-N. and Maloteaux, J.-M. (1996) Interaction of the COOH-terminal domain of the neurotensin receptor with a G protein does not control the phospholipase C activation but is involved in agonist-induced internalization. Mol. Pharmalcol. 49, 365-372.

Hertel, C., Coulter, S.J. and Perkins, J.P. (1985) A comparison of catecholamine-induced internalization of β-adrenergic receptors and receptor-mediated endocytosis of epidermal growth factor in human astrocytoma cells. Inhibition by phenylarsine oxide. J. Biol. Chem. 260, 12547-12553.

Hicke, L. and Reizman, H. (1996) Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis. Cell 84, 277-287.

Hicke, L., Zanolori, B. and Reizman, H. (1998) Cytoplasmic tail phosphorylation of the α-factor receptor is required for its ubiquitination and internalization. J. Cell Biol. 141, 349-358.

Hinshaw, J.E. and Schmid, S.L. (1995) Dynamin sel-assembles into rings suggesting a mechanism for coated vesicle budding. Nature 374, 190-192.

Hoelscher, S.R., Sairam, M.R. and Ascoli, M. (1991) The slow rate of internalization of deglycosylated hCG is not due to its inability to stimulate cAMP accumulation. Endocrinology 128, 2837-2843.

Holtmann, M.H., Roettger, B.F., Pinon, D.I. and Miller, L.J. (1996) Role of receptor phosphorylation in desensitization and internalization of the secretin receptor. J. Biol. Chem. 271, 23566-23571.

Hopkins, C.R. and Gregory, H. (1977) Topographical localization of the receptors for luteinizing hormone-releasing hormone on the surface of dissociated pituitary cells. J. Cell. Biol. 75, 528-540.

Hoxie, J.A., Ahuja, M., Belmonte, E., Pizarro, S., Parton, R. and Brass, L.F. (1993) Internalization and recycling of activated thrombin receptors. J. Biol. Chem. 268, 13756-13763.

Huang, S., Chem, Y. and Nissenson, R.A. (1995) The cytoplasmic tail of the G protein-coupled receptor for parathyroid hormone and parathyroid hormone-related protein contains positive and negative signals for endocytosis. J. Biol. Chem. 270, 151-156.

Hukovic, N., Panetta, R., Kumar, U., Rocheville, M. and Patel, Y.C. (1998) The cytoplasmic tail of the human somatostatin receptor type 5 is crucial for interaction with adenylyl cyclase and in mediating desensitization and internalization. J. Biol. Chem. 273, 21416-21422.

Hunyady, L., Baukal, A.J., Balla, T. and Catt, K.J. (1994a) Independence of type I angiotensin II receptor endocytosis from G protein coupling and signal transduction. J. Biol. Chem. 269, 24798-24804.

Hunyady, L., Bor, M., Balla, T. and Catt, K.J. (1994b) Identification of a cytoplasmic Ser-Thr-Leu motif that determines agonist-induced internalization of the AT_1 angiotensin receptor. J. Biol. Chem. 269, 31378-31382.

Hunyady, L., Tian, Y., Sandberg, K., Balla, T. and Catt, K.J. (1994c) Divergent conformational requirements for angiotensin II receptor internalization and signaling. Kidney. Int. 46, 1496-1498.

Hunyady, L., Bor, M., Baukal, A.J., Balla, T. and Catt, K.J. (1995) A conserved NPLFY sequence contributes to agonist binding and signal transduction but is not an internalization signal for the type 1 angiotensin II receptor. J. Biol. Chem. 270, 16602-16609.

Illing, N., Jacobs, G.F., Becker, I.I., Flanagan, C.A., Davidson, J.S., Eales, A., Zhou, W., Sealfon, S.C. and Millar, R.P. (1993) Comparative sequence analysis and functional characterization of the cloned sheep gonadotropin-releasing hormone receptor reveal differences in primary structure and ligand specificity

among mammalian receptors. Biochem. Biophys. Res. Commun. 196, 745-751.

Innamorati, G., Sadeghi, H. and Birnbaumer, M. (1998) transient phosphorylation of V1a vasopressin receptor. J. Biol. Chem. 273, 7155-7161.

Itokawa, N., Toru, M., Ito, K., Tsuga, H., Kameyama, K., Haga, T., Arinami, T. and Hamaguchi, H. (1996) Sequestration of the short and long isoforms of dopamine D_2 receptors expressed in Chinese hamster ovary cells. Mol. Pharmacol. 49, 560-566.

Ishii, K., Chen, J., Ishii, M., Koch, W.J. and Freedman, N.J. (1994) Inhibition of hrombin receptor signaling by a G protein-coupled receptor kinase: functional specificity among G protein-coupled receptor kinases. J. Biol. Chem. 269, 1125-1130.

Ishii, I., Saito, E., Izumi, T., Ui, M. and Shimizu, T. (1998) Agonist-induced sequestration, recycling, and resensitization of platelet-activating factor receptor. Role of cytoplasmic tail phosphorylation in each process. J. Biol. Chem. 273, 9878-9885.

Iwashita, M. and Catt, K.J. (1985) Photoaffinity labeling of piuitary and gonadal receptors for gonadotropin-releasing hormone. Endocrinology 117, 738-746.

Jennes, L., Stumpf, W.E. and Conn, P.M. (1983) Intracellular pathways of electron-opaque gonadotropin-releasing hormone derivatives bound by cultured gonadotropes. Endocrinology 113, 1683-1689.

Jennes, L., Coy, D. and Conn, P.M. (1986) Reptor-mediated uptake og GnRH agonist and antagonists by cultured gonadotropes: evidence for differentail intracellular routing. Peptides 7, 459-463.

Jewell-Motz, E. and Liggett, S.B. (1996) G protein-coupled receptor kinase specificity for phosphorylation and desensitization of α_2-adrenergic receptor subtypes. J. Biol. Chem. 271, 18082-18087.

Jones, P.G., Curtis, C. A.M. and Hulme, E.C. (1995) The fuction of a highly conserved arginine residue in activation of the muscarinic M_1 receptor. Eur. J. Pharmacol. 288, 251-257.

Kaiser, U.B., Zhao, D., Cardona, G.R. and Chin, W.W. (1992) Isolation and characterization of cDNAs encoding the rat pituitary gonadotropin-releasing hormone receptor. Biochem. Biophys. Res. Commun. 189, 1645-1652.

Kakar, S.S., Musgrove, L.C., Devor, D.C., Sellers, J.C. and Neill, J.D. (1992) Cloning, sequencing, and expression of human gonadotropin releasing hormone (GnRH) receptor. Biochem. Biophys. Res. Commun. 189, 289-295.

Kakar, S.S., Rahe, C.H. and Neill, .D. (1993) Molecular cloning, sequencing, and characterizing the bovine receptor for gonadotropin releasing hormone (GnRH). Domest. Anim. Endocrinol. 10, 335-342.

Kenakin, T. (1993) Pharmacologic Analysis of Drug-Receptor Interaction. 2nd Ed., pp. 39-68, Raven Press, New York.

Koch, T., Schulz, S., Schroder, H., Wolf, R., Raulf, E. and Hollt, V. (1998) Carboxyl-terminal splicing of the rat mu opioid receptor modulates agonist-mediated internalization and receptor resensitization. J. Biol. Chem. 273, 13652-13657.

Kong, G., Penn, R. and Benovic, J.L. (1994) A β-adrenergic receptor kinase dominant negative mutant attenuates desensitization of the β_2-adrenergic receptor. J. Biol. Chem. 269, 13084-13087.

Krupnick, J.G., Goodman, Jr., O.B., Keen, J.H. and Benovic, J.L. (1997a) Arrestin/clathrin interaction: Localization of the clathrin binding domain of nonvisual arrestins to the carboxyl terminus. J. Biol. Chem. 272, 15011-15016.

Krupnick, J.G., Santini, F., Gagnon, A.W., Keen, J.H. and Benovic, J.L. (1997b) Modulation of arrestin-clathrin interaction in cells: charaterization of β-arrestin dominant-negative mutants. J. Biol. Chem. 272, 32507-32512.

Krupnick, J.G. and Benovic, J.L. (1998) The role of receptor kinases and arrestins in G protein-coupled receptor regulation. Annu. Rev. Pharmacol. Toxicol. 38, 289-319.

Kunkel, T.A., Bebenek, K. and McClary, J. (1991) 1991 Efficient site-directed mutagenesis using uracil-containing DNA. Methods Enzymol. 204, 125-139.

Kwatra, M.M., Schwinn, D.A., Schreurs, J., Blank, J.L., Kim, C.M., Benovic, J.L., Krause, J.E., Caron, M.G. and Lefkowitz, R.J. (1993) The substance P receptor, which couples to $G_{q/11}$, is a substrate for β-adrenergic receptor kinase 1 and 2. J. Biol. Chem. 268, 9161-9164.

Lamaze, C., Chuang, T.-H., Terlecky, L.J., Bokoch, G.M. and Schmid, S.L. (1996) Regulation of receptor-mediated endocytosis by rho and rac. Nature 382, 177-179.

Lameh, J., Philip, M., Sharma, Y.K., Moro, O., Ramachandran, J. and Sadée, W. (1992) Hm1 muscarinic cholinergic receptor internalization requires a domain in the third cytoplasmic loop. J. Biol. Chem. 267, 13406-13412.

Laporte, S.A., Servant, G., Richard, D.E., Escher, E., Guillemette, G. and Leduc, R. (1996) The tyrosine within the NPX_nY motif of the human angiotensin II type 1 receptor is involved in mediating signal transduction but is not essential for internalization. Mol. Pharmacol. 49, 89-95.

Lattion, A.L., Diviani, D. and Cotecchia, S. (1994) Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitization of the α_{1B}-adrenergic receptor. J. Biol. Chem. 269, 22887-22893.

Lazari, Maria de Fatima M., Bertrand, J.E., Nakamura, K., Liu, X., Krupnick, J.G., Benovic, J.L. and Ascoli, M. (1998) Mutation of indiviual serine residues in the c terminal tail of the lutropin/choriagonadotropin receptor reveal distinct structural requirements for agonist-induced uncoupling and agonist-induced internalization. J. Biol. Chem. 273, 18316-18324.

Le Gouill, C., Parent, J-L., Rola-Pleszczynski, M. and Stankova, J. (1997) Structural and functional requirements for agonist-induced internlaization of the human platelet-activating factor receptor. J. Biol. Chem. 272, 21289-21295.

Lee, K.B., Pals-Rylaarsdam, R., Benovic, J.L. and Hosey, M.M. (1998) Arrestin-independent internalization of the m1, m3, and m4 subtypes of muscarinic cholinergic receptors. J. Biol. Chem. 273, 12967-12972.

Leeb-Lundberg, L.M.F., Cotecchia, S., Lomasney, J., DeBernardis, J.F., Lefkowitz, R.J. and Caron, M.G. (1985) Phorbol esters promote α_1-adrenergic receptor phosphorylation and receptor uncoupling from inositol phospholipid metabolism. Proc. Natl. Acad. Sci. USA 82, 5651-5655.

Lefkowitz, R.J., Stadel, J.M. and Caron, M.G. (1983) Adenylate cyclase-coupled β-adrenergic receptors: structure and mechanisms of activation and desensitization. Ann. Rev. Biochem. 52, 159-186.

Lefkowitz, R.J. and Caron, M.G. (1986) Regulation of adrenergic receptor function by phosphorylation. Curr. Top. Cell. Reg. 28, 209-231.

Lefkowitz, R.J. and Caron, M.G. (1987) Molecular and regulatory properties of adrenergic receptors. Rec. Prog. Horm. Res. 43, 469-497.

Lefkowitz, R.J. (1993) G protein-coupled receptor kinases. Cell 74, 409-412.

Leung, D.W., Spencer, S.A., Cachianes, G., Hammonds, R.G., Collins, C., Henzel, W.J., Barnard, R., Waters, M.J. and Wood, W.I. (1987) Growth hormone receptor and serum binding protein: purification, cloning and expression. Nature. 330, 537-543.

Li, S.L., Vuagnat, B., Gruaz, N.M., Eshkol, A., Sizonenko, P.C. and Aubert, M.I. (1994) Binding kinetics of the long acting gonadotropin-releasing hormone (GnRH) antagonist antide to rat pituitary GnRH receptor. Endocrinology 134, 45-52.

Li, S., Okamoto, T., Chun, M., Sargiacomo, M., Casanova, J.E., Hansen, S.H., Nishimoto, I. and Lisanti, M.P. (1995) Evidence for a regulated interaction between heterotrimeric G proteins and caveolin. J. Biol. Chem. 270, 15693-15701.

Liang, M., Eeason, M.G., Jewell-Motz, E.A., Williams, M.A., Theiss, C.T., Dorn, G.W. and Liggett, S.B. (1998) Phosphorylation and functional desensitization of the α_{2A}-adrenergic receptor by protein kinase C. Mol. Pharmacol. 54, 44-49.

Liggett, S.B., Ostrowski, J., Chesnut, L.C., Kurose, H., Raymond, J.R., Caron, M.G. and Lefkowitz, R.J. (1992) Sites in the third intracellular loop of the α_{2A}-adrenergic receptor confer short term agonist-promoted desensitization. J. Biol. Chem. 267, 4740-4746.

Lin, F.-T., Krueger, K.M., Kendall, H.E., Daaka, Y., Fredericks, Z.L., Pitcher, J.A. and Lefkowitz, R.J. (1997) Clathrin-mediated endocytosis of the β-adrenergic receptor is regulated by phosphorylation/dephosphorylation of β-arrestin-1. J. Biol. Chem. 272, 31051-31057.

Lin, X., Janovick, J.A., Brother, S., Blomenrohr, M., Bogerd, J. and Conn, P.M. (1998) Addition of catfish gonadotropin-releasing hormone (GnRH) receptor intracellular carboxyl-terminal tail to rat GnRH receptor alters receptor expression and regulation. Mol Endocrinol. 12, 161-171.

Liu, J.-P., Yajima, Y., Li, H., Ackland, S., Akita, Y., Stewart, J. and Kawashima, S. (1997) Molecular interactions between dynamin and G protein βγ-subunits in neuroendocrine cells. Mole. Cell Endocrinol. 132, 61-71.

Loeb, J.N. and Strickland, S. (1987) Hormone binding and coupled response relationships in systems dependent on the generation of secondary mediators. Mol. Endocrinol.1, 75-82.

Loumaye, E., Wynn, P.C., Coy, D. and Catt, K.J. (1984) Receptor-binding properties of gonadotropin-releasing hormone derivatives: prolonged receptor

occupancy and cell-surface localization of a potent antagonist analog. J. Biol. Chem. 259, 12663-12671.

Lutz, W., Londowski, J.M., Sanders, M., Salisbury, J. and Kumar, R. (1992) A vasopressin analog that binds but does not activate V1 or V2 vasopressin receptors is not internalized into cells that express V1 or V2 receptors. J. Biol. Chem. 267, 1109-1115.

Lynch, C.J., Taylor, S.J., Smith, J.A. and Exton, J.H. (1988) Formation of the high-affinity agonist state of the α_1 -adrenergic receptor at cold temperatures does not require a G protein. FEBS Lett. 229, 54-58.

Maeda, S., Lameh, J., Mallet, W.G., Philips, M., Ramachandran, J. and Sadée, W. (1990) Internalization of the Hm1 muscarinic cholinergic receptor involves the third intracellular loop. FEBS. Lett. 269, 386-388.

Madaule, P. and Axel, R. (1985) A novel ras-related gene family. Cell 41, 31-40.

Mason, D.R., Arora, K.K., Mertz, L.M. and Catt, K.J. (1994) Homologous down-regulation of gonadotropin-releasing hormone receptor sites and messenger ribonucleic acid transcripts in αT3-1 cells. Endocrinology 135, 1165-1170.

McArdle, C.A., Bunting, R. and Mason, W.T. (1992) Dynamic video imaging of cytosolic Ca^{2+} in the αT3-1, gonadotrope-derived cell line. Mol. Cell. Neurosci. 3, 124-132.

McArdle, C.A., Forrest-Owen, W., Willars, G., Davidson, J.S., Poch, A. and Kratzmeier, M. (1995) Desensitization of gonadotropin-releasing hormone action in the gonadotrope-derived αT3-1 cell line. Endocrinol. 136, 4864-4871.

McArdle, C.A., Willars, G.B., Fowkes, R.C., Nahorski, S.R., Davidson, J.S. and Forrest-Owen, W. (1996) Desensitization of gonadotropin-releasing hormone action in αT3-1 cells due to uncoupling of inositol 1,4,5-trisphosphate generation and Ca^{2+} mobilization.

McConalogue, K., Corvera, C.U., Gamp, P.D., Grady, E.F. and Bunnett, N.W. (1998) Desensitization of the neurokinin-1 receptor (NK1-R) in neurons: effects of substance P on the the distribution of NK1-R, $G\alpha_{q/11}$, GRK2/3, and β-arrestins-1/2

Ménard, L., Ferguson, S.S.G., Barak, L.S., Bertrand, L., Premont, R.T., Colapietro, A. -M., Lefkowitz, R.J. and Caron, M.G. (1996) Members of the G protein-coupled receptor kinase family that phosphorylate the β_2-adrenergic receptor facilitate sequestration. Biochemistry 35, 4155-4160.

Mellman, I. (1996) Endocytosis and molecular sorting. Annu. Rev. Cell Dev. Biol. 12, 575-625.

Milici, A.J., Watrous, N.E., Stukenbrok, H. and Palade, G.E. (1987) Transcytosis of albumin in capilary endothelium. J. Cell. Biol. 105, 2603-2612.

Millar, R.P. and King, J.A. (1984) Structure-activity relations of LH-RH in birds. J. Exp. Zoo.232, 425-430.

Millar, R.P., King, J.A., Davidson, J.S. and De L. Milton, R.C. (1987) Gonadotropin-releasing hormone - diversity of functions and clinical applications. S. Afr. Med. J. 72, 748-755.

Min, K.C., Zvyaga, T.A., Cypress, A.M. and Sakmar, T.P. (1993) Characterization of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa. J. Biol. Chem. 268, 9400-9404.

Moro, O., Lameh, J. and Sadée, W. (1993) Serine- and Threonine-rich domain regulates internalization of muscarinic cholinergic receptors. J. Biol. Chem. 268, 6862-6865.

Moore, R.H., Sadovnikoff, N., Hoffenberg, S., Shaobin, L., Woodford, P., Angelides, K., Trial, J., Carsrud, N.D.V., Dickey, B.F. and Knoll, B.J. (1995) Ligand-stiulated β_2-adrenergic receptor internalization via the constitutive endocytic pathway into ran5-containing endosomes. J. Cell Sci. 108, 2983-2991.

Muhlberg, A.B., Marnock, D.E. and Schmid, S.L. (1997) Domain structure and intramolecular regulation of dynamin GTPase. EMBO J. 16, 6676-6683.

Mundell, S.J., Benovic, J.L. and Kelly, E. (1997) A dominant negative mutant of thje G protein-coupled receptor kinase 2 selectively attenutates adenosine A2 receptor desensitization. Mol. Pharmacol. 51, 991-998.

Munson, P.J. and Rodbard, D. (1988) An exact correction to the "Cheng-Prusoff" correction. J. Receptor Res. 8, 533-546.

Myburgh, D.B., Millar, R.P. and Hapgood, J.P. (1998) Alanine-261 in intracellular loop III of the human gonadotropin-releasing hormone receptor is crucial for G protein-coupling and receptor internalization. Biochem. J. 331, 893-896.

Nakamura, K., Hipkin, R.W. and Ascoli, M. (1998) The agonist-induced phosphorylation of the rat follitropin receptor maps to the first and third intracellular loops. Mol. Endocrinol. 12, 580-591.

Nakata, H., Kameyama, K., Haga, K. and Haga, T. (1994) Location of the agonist-dependent phosphorylation sites in the third intracellular loop of muscarinic acetylcholine receptors (m2 subtype). Eur. J. Biochem. 220, 29-36.

Naor, Z., Atlas, D., Clayton, R.N., Forman, D.S., Amsterdam, A. and Catt, K.J. (1981) Interaction of fluorescent gonadotropin-releasing hormone with receptors in cultured pituitary cells. J. Biol. Chem. 256, 3049-3052.

Neill, J.D., Duck, L.W., Sellers, J.C., Musgrove, L.C., Scheschonka, A., Druey, K.M. and Kehrl, J.H. (1997) Potential role for a regulator of G protein signaling (RGS3) in gonadotropin-releasing hormone (GnRH) stimulated desensitization. Endocrinology 138, 843-846.

Neill, J.D., Duck, L.W., Musgrove, L.C. and Sellers, J.C. (1998) Potential regulatory role for G protein-coupled receptors kinases and β-arrestins in gonadotropin-releasing hormone receptor signaling. Endocrinology 139, 1781-1788.

Nussenzveig, D.R., Heinflink, M. and Gershengorn, M.C. (1993a) Agonist-stimulated internalization of the thyrotropin-releasing hormone receptor is dependent on two domains in the receptor carboxyl terminus. J. Biol. Chem. 268, 2389-2392.

Nussenzveig, D.R., Heinflink, M. and Gershengorn, M.C. (1993b) Decreased levels of internalized thyrotropin-releasing hormone receptors after uncoupling from guanine nucleotide-binding protein and phospholipase-C. Mol. Endocrinol. 7, 1105-1111.

Ohno, H., Stewart, J., Fournier, M.C., Bosshart, H., Rhee, I, Miyatake, S., Saito, T., Gallusser, A., Kirchhausen, T. and Bonifacino, J.S. (1995) Interaction of tyrosine-based sorting signals with clathrin associated proteins. Science 269, 1872-1875.

Ohyama, K., Yamano, Y., Chaki, S., Kondo, T. and Inagami, T. (1992) Domains for G protein-coupling in angiotensin II receptor type I: studies by site-directed mutagenesis. Biochem. Biophys. Res. Commun. 189, 677-683.

Oliveira, L., Paiva, A.C.M., Sander, C. and Vriend, G. (1994) A common step for signal transduction in G-protein-coupled receptors. TIPS 15, 170-172.

Oppermann, M., Freedman, N.J., Alexander, R.W. and Lefkowitz, R.J. (1996) Phosphorylation of the type 1A angiotensin II receptor by G protein-coupled receptor kinases and protein kinase C. J. Biol. Chem. 271, 13266-13272.

Ozcelebi, F., Rao, R.V., Holicky, E., Madden B.J., McCormick, D.J. and Miller L.J. (1996) Phosphorylation of cholecytokinin receptors expressed on Chinese hamster ovary cells. J. Biol. Chem. 271, 3750-3755.

Palcezewski, K. (1994) Structure and function of arrestins. Protein Sci. 3, 1355-1361.

Palmer, T.M., Benovic, J.L. and Stiles, G.L. (1995) Agonist-dependent phosphorylation and desensitization of the rat A3 adenosine receptor: evidence for a G protein-coupled receptor kinase-mediated mechanism. J. Biol. Chem. 270, 29607-29613.

Pals-Rylaarsdam, R., Xu, Y., Witt-Enderby, P., Benovic, J.L. and Hosey, M.M. (1995) Desensitization and internalization of the m2 muscarinic acetylcholine receptor are directed by independent mechanisms. J. Biol. Chem. 270, 29004-29011.

Pals-Rylaarsdam, R., Gurevich, V.V., Lee, K.B., Ptasienski, J.A., Benovic, J.L. and Hosey,M.M. (1997) Internalization of the m2 muscarinic acetylcholine receptor: arrestin-indepedent and -dependent pathways. J. Biol. Chem. 272, 23682-23689.

Pawson, A.J., Katz, A., Sun, Y.-M., Illing, N., Lopes, J., Millar, R.P. and Davidson, J.S. (1998) Contrasting internalization kinetics of human and chicken gonadotropin-releasing hormone receptors mediated by C-terminal tail. J. Endocrinol. 156(3), R9-R12.

Pearse, B.M.F. (1988) Receptors compete for adaptors found in plasma membrane coated pits. EMBO J. 7, 3331-3336.

Pearse, B.M.F. and Robinson, M.S. (1990) Clathrin, adaptors, and sorting. Annu Rev. Cell Biol. 6, 151-171.

Pei, G., Kieffer, B.L., Lefkowitz R.J. and Freedman, N.J. (1995) Agonist-dependent phosphorylation of the mouse δ-opiod receptor: involvement of G protein-coupled receptor kinases but not protein kinase C. Mol. Pharmacol. 48, 173-177.

Pelletier, G., Dube, D., Guy, J., Seguin, C. and Lefebvre, F.A. (1982) Binding and internalization of a luteinizing hormone-releasing hormone receptor agonist by rat gonadotrope cells. A radiographic study. Endocrinology 111, 1069-1076.

Peters, K.R., Carley, W.W. and Palade, G.E. (1985) Endothelial plasmalemmal vesicles have a characteristic striped bipolar surface structure. J. Cell. Biol. 101, 2233-2238.

Pfeiffer, R., Kirsch, J. and Fahrenholz, F. (1998) Agonist and Antagonist-Dependent Internalization of the Human Vasopressin V2 Receptor. Exp. Cell. Res. 10, 327-339

Pippig, S., Andexinger, S., Daniel, K., Puzicha, M., Caron, M.G., Lefkowitz, R.J. and Lohse, M.J. (1993) Overexpression of β-arrestin and β-adrenergic receptor kinase augment desensitization of β_2-adrenergic receptors. J. Biol. Chem. 268, 3201-3208.

Pippig, S., Andexinger, S. and Lohse, M.J. (1995) Sequestration and recycling of β_2-adrenergic receptors permit receptor resensitization. Mol. Pharmacol. 47, 666-676.

Pitcher, J.A., Inglese, J., Higgins, J.B., Arriza, J.L., Casey, P.J., Kim, C., Benovic, J.L., Kwatra, M.M., Caron, M.G. and Lefkowitz, R.J. (1992) Role of βγ subunits of G proteins in targeting the β-adrenergic receptor kinase to membrane-bound receptors. Science 257, 1264-1267.

Pitcher, J.A., Hall, R.A., Daaka, Y., Zhang, J., Ferguson, S.S.G., Hester, S., Miller, S., Caron, M.G., Lefkowitz, R.J. and Barak, L.S. (1998) The G protein-coupled receptor kinase 2 is a microtubule-associated protein kinase that phosphorylates tubulin. J. Biol. Chem. 273, 12316-12324.

Polakiewicz, R.D., Schieferl, S.M., Dorner, L.F., Kansra, V. and Comb, M.J. (1998) A mitogen-activated protein kinase pathway is required for μ-opiod receptor desensitization. J. Biol. Chem. 273, 12402-12406.

Prado, G.N., Suzuki, H., Wilkinson, N., Cousins, B. and Navarro, J. (1996) Role of the C terminus of the interleukin 8 receptor in signal transduction and internalization. J. Biol. Chem. 271, 19186-19190.

Probst, W.C., Snyder, L.A., Schuster, D.I., Brosius, J. and Sealfon, S.C. (1992) Sequence alignment of the G protein-coupled receptor superfamily. DNA Cell Biol. 11, 1-29.

Raynor, K., Kong, H., Hines, J., Kong, G., Benovic, J., Yasuda, K., Bell., G.I. and Reisine, T. (1994) Molecular mechanisms of agonist-induced desensitization of the cloned mouse κ-opiod receptor. J. Pharmacol. Exp. Ther. 270, 1381-1386.

Reinhart, J., Mertz, L.M. and Catt, K.J. (1992) Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. J. Biol. Chem. 267, 21281-21284.

Reizman, H., Woodman, P.G., van Meer, G. and Marsh, M. (1998) Molecular mechanisms of endocytosis. Cell 91, 731-738.

Reneke, J.E., Blumer, K.J., Courchesne, W.E. and Thorner, J. (1988) The carboxy-terminal segment of the yeast alpha-factor receptor is a regulatory domain. Cell. 55, 221-234.

Richardson, R.M., Ali, H., Pridgen, B.C., Haribabu, B. and Snyderman, R. (1998) Multiple signaling pathways of human interleukin-8 receptor A. J. Biol. Chem. 273, 10690-10695.

Rodriguez, M.C., Xie, Y-B., Wang, H., Collision, K. and Segaloff, D.L. (1992) Effects of truncations of the cytoplasmic tail of the luteinizing hormone/chorionic gonadotropin receptor on receptor-mediated internalization. Mol. Endocrinol. 6, 327-336.

Roettger, B.F., Rentsch, R.U., Pinon, D., Holicky, E., Hadac, E.M., Larkin, J.M and Miller, L.J. (1995) Dual pathways of internalization of the cholecystokinin receptor. J. Cell Biol. 128, 1029-1041.

Roettger, B.F., Ghanekar, D., Rao, R., Toledo, C., Yingling, J., Pinon, D. and Miller, L.J. (1997) Antagonist-stimulated internalization of the G protein-coupled cholecustokinin receptor. Mol. Pharmacol. 51, 357-362.

Rosenthal, W., Antaramian, A., Gilbert, S. and Birnbaumer, M. (1993) Nephrogenic diabetes insipidus. A V2 vasopressin receptor unable to stimulate adenylyl cyclase. J. Biol. Chem. 268, 13030-13033.

Ruiz-Gómez, A. and Mayor, Jr., F. (1997) β-adrenergic receptor kinase (GRK2) colocalizes with β-adrenergic receptors during agonist-induced internalization. J. Biol. Chem. 272, 9601-9604.

Sakmar, T.P, Franke, R.R. and Khorana, H.G. (1989) Glutamic acid-113 serves as the retinylidene Schiff base counterion in bovine rhodopsin. Proc. Natl. Acad. Sci. U.S.A. 86, 8309-8313.

Sánchez-Yague, J., Rodriguez, M.C., Segaloff, D.L. and Ascoli, M. (1992) Truncation of the cytoplasmic tail of the lutropin/choriogonadotropin receptor prevents agonist-induced uncoupling. J. Biol. Chem 267, 7217-7220.

Sanger, F., Niklen, S. and Coulson, A.R. (1977) DNA sequencing with chain-termination inhibitors. Proc. Natl. Acad. Sci. USA 74,5463-5467.

Savarese, T.M. and Fraser, C.M. (1992) *In vitro* mutagenesis and the search for structure-function releationships among G protein-coupled receptors. Biochem. J. 283, 1-19.

Schagger, H. and von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for separation of proteins in the range 1 to 100 kDa. Analytical Biochemistry 166, 368-379.

Scheer, A., Fanelli, F., Costa, T., De Benedetti, P.G. and Cotecchia, S. (1996) Constitutively active mutants of the α_{1b}-adrenergic receptor: role of highly conserved polar amino acids in receptor activation. EMBO J. 15, 3566-3578.

Schetzline, M.A., Premont, R.T., Walker, J.K.L., Vigna, S.R. and Caron, M.G. (1998) A role for receptor kinases in the regulation of class II G protein-coupled receptors. J. Biol. Chem. 273, 6756-6762.

Schlador, M.L. and Nathanson, N.M. (1997) Synergistic regulation of m2 muscarinic acetylcholine receptor desensitization and sequestration by G protein-coupled receptor kinase-3 and β-arrestin-1. J. Biol. Chem. 272, 18882-18890.

Schmalzing, G., Richter, H.-P., Hansen, A., Schwarz, W., Just, I. and Aktories, K. (1995) Involvement of the GTP-binding protein rho in constitutive endocytosis in *Xenopus laevis* oocytes. J. Cell. Biol. 130, 1319-1332.

Schvartz, I. and Hazum, E. (1987) Internalization and recycling of receptor-bound gonadotropin-releasing hormone agonist in pituitary gonadotropes. J. Biol. Chem. 262, 17046-17050.

Schwarz, K.R., Lanier, S.M., Sena, L.M., Carter, E.A., Graham, R.M. and Homey, C.J. (1986a) Agonist-induced isomerization of the α_1 -adrenergic receptor: kinetic analysis using broken-cell and solubilized preparations. Biochemistry 25, 2697-2702.

Schwarz, K.R., Carter, E.A., Homey, C.J. and Graham, R.M. (1986b) Agonist interactions at hepatic α_1 - and β-adrenergic receptors: affinity-state regulation by guanine nucleotides and temperature. Biochemistry 25, 7782-7788.

Sealfon, S.C., Weinstein, H. and Millar, R.P. (1997) Molecular mechanisms of ligand interaction with the gonadotropin-releasing hormone receptor. Endocr. Rev. 18, 180-205.

Seibold, A., January, B.G., Friedman, J., Hipkin, R.W. and Clark, R.B. (1998) Desensitization of β_2-adrenergic receptors with mutantions of the proposed C-protein coupled receptor kinase phosphorylation sites. J. Biol. Chem. 273, 7637-7642.

Shapiro, M.J., Trejo, J., Zeng, D. and Coughlin, S.R. (1996) Role of the thrombin receptor's cytoplasmic tail in intracellular trafficking. J. Biol. Chem. 271, 32874-32880.

Sibley, D.R., Nambi, P., Peters, J.R. and Lefkowitz, R.J. (1984) Phorbol diesters promote β-adrenergic receptor phosphorylation and adenylate cyclase desensitization in duck erythrocytes. Biochem. Biophys. Res. Comm. 121, 973-979.

Sibley, D.R., Strasser, R.H., Benovic, J.L., Daniel, K. and Lefkowitz, R.J. (1986) Phosphorylation/dephosphorylation of the β-adrenergic receptor regulates its functional coupling to adenylate cyclase and subcellular dirtubution. Proc. Natl. Acad. Sci. USA. 83, 9408-9412.

Sibley, D.R., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. (1987) Regulation of transmembrane signaling by receptor phosphorylation. Cell 48, 913-922.

Sibley, D.R., Benovic, J.L., Caron, M.G. and Lefkowitz, R.J. (1988) Phosphorylation of cell surface receptor: a mechanism for regulating signal transduction pathways. Endocr. Rev. 9, 38-56.

Slice, L.W., Wong, H.C., Sternini, C., Grady, E.F., Bunnett, N.W. and Walsh, J.H. (1994) The conserved NPX_nY motif present in the gastrin-releasing peptide receptor is not a general sequestration sequence. J. Biol. Chem. 269, 21755-21762.

Stadel, J.M., Nambi, P., Shorr, R.G.L., Sawyer, D.F., Caron, M.G. and Lefkowitz, R.J. (1983) Catecholamine-induced desensitization of turkey erythrocyte adenylate cyclase is associated with phosphorylation of the β-adrenergic receptor. Proc. Natl. Acad. Sci. USA 80, 3173-3177.

Sterne-Marr, R. and Benovic, J.L. (1995) Regulation of G protein-coupled receptors by receptor kinases and arrestins. Vitam. Horm. 51, 193-234.

Stojilkovic, S.S. and Catt, K.J. (1992) Calcium oscillations in anterior pituitary cells. Endocr. Rev. 13, 256-280.

Stojilkovic, S.S., Reinhart, J. and Catt, K.J. (1994) Gonadotropin-releasing hormone receptor: Structure and signal transduction pathways. Endocr. Rev. 15, 462-499.

Stojilkovic, S.S. and Catt, K.J. (1995) Expression and signal transduction pathways of gonadotropin-releasing hormone receptors. Rec. Prog. Hor. Res. 50, 161-205.

Stoorvogel, W., Oorschot, V. and Geuze, H.J. (1996) A novel class of clathrin-coated vesicles budding from endosomes. J. Cell Biol. 132, 21-33.

Strader, C.D., Sigal, I.S., Blake, A.D., Cheung, A.H., Register, R.B., Rands, E., Zemcik, B.A., Candelore, M.R. and Dixon, R.A. (1987) The carboxyl terminus of the hamster beta-adrenergic receptor expressed in mouse L-cells is not required for receptor sequestration. Cell 49, 855-863.

Strader, C.D., Fong, T.M., Tota, M.R., Underwood, D. and Dixon, R.A.F. (1994) Structure and function of G protein-coupled receptors. Annu. Rev. Biochem. 63, 101-132.

Strous, G., van Kerkhof, P., Govers, R., Ciechanover, A. and Schwartz, A.L. (1996) The ubiquitin conjugation system for ligand-induced endocytosis and degradation of the growth hormone receptor. EMBO J. 15, 3806-3812.

Suarez-Quian, C.A., Wynn, P.C. and Catt, K.J. (1986) Receptor-mediated endocytosis of GnRH analogs: differential processing of gold-labeled agonist and antagonist derivatives. J. Steroid. Biochem. 24, 183-192.

Takei, K., McPherson, P.S., Schmid, S.L. and De Camilli, P. (1995) Tubular membrane invaginations coated by dynamin rings are induced by GTPγS in nerve terminals. Nature 376, 186-189.

Tensen, C., Okuzawa, K., Bloenröhr, M., Rebers, F., Leurs, R., Bogerd, I., Schulz, R. and Goos, H. (1997) Distinct efficacies for two endogenous ligands on a single gonadoliberin receptor. Eur. J. Biochem. 243, 134-140.

Thilo, L., Stroud, E. and Haylett, T. (1995) Maturation of early endosomes and vesicular traffic to lysosomes in relation to membrane recycling. J. Cell Science 108, 1791-1803.

Thomas, W.G., Thekkumkara, T.J., Motel, T.J. and Baker, K.M. (1995a) Stable Expression of a Truncated AT Receptor in CHO-K1 Cells. J. Biol. Chem. 270, 207-213.

Thomas, W.G., Baker, K.M., Motel, T.J. and Thekkumkara, T.J. (1995b) Angiotensin II receptor endocytosis involves two distinct regions of the cytoplasmic tail. A role for residues on the hydrophobic face of a putative amphipathic helix. J. Biol. Chem. 270, 22153-22159.

Thompson, A.K., Mostafapour, S.P., Denlinger, L.C., Bleasdale, J.E. and Fisher, S.K. (1991) The aminosteroid U-73122 inhibits Muscarinic receptor sequestration and phosphoinositide hydrolysis in SK-N-SH neuroblastoma cells. J. Biol. Chem. 266, 23856-23862.

Tobin, A.B., Lambert, D.G. and Nahorski, S.R. (1992) Rapid desensitization of muscarinic m3 receptor-stimulated polyphosphoinositide responses. Mol. Pharmacol. 42, 1042-1048.

Tobin, A.B. and Nahorski, S.R. (1993) Rapid agonist-mediated phosphorylation of m3 muscarinic receptors revealed by immunoprecipitation. J. Biol. Chem. 268, 9817-9823.

Tobin, A.B., Keys, B. and Nahorski, S.R. (1996) Identification of a novel receptor kinase that phosphorylates a phospholipase C-linked muscarinic receptor. J. Biol. Chem. 271, 3907-3916.

Tolbert, L.M. and Lameh, J. (1996) Human muscarinic cholinergic receptor Hm1 internalizes via clathrin-coated vesicles. J. Biol. Chem. 271,17335-17342.

Trapaidze, N., Keith, D.E., Cvejic, S., Evans, C.J. and Devi, L.A. (1996) Sequetration of the δ opiod receptor. J. Biol. Chem. 271, 29279-29285.

Troskie, B.E., Sun, Y., Hapgood, J., Sealfon, S.C., Illing, N. and Millar, R.P. (1997) Mammalian GnRH receptor functional features revealed by comparative sequences of goldfish, frog and chicken receptors. Program of the 79th Annual Meeting of The Endocrine Society, Minneapolis, Minnesota, p1-130 (Abstract).

Trowbridge, I.S. (1991) Endocytosis and signals for internalization. Curr. Op. Cell Biol. 3, 634-641.

Trowbridge, I.S., Collawn, J.F. and Hopkins, C.R. (1993) Signal-dependent membrane protein trafficking in the endocytic pathway. Annu. Rev. Cell Biol. 9, 129-161.

Trumpp-Kallmeyer, S., Hoflack, J., Bruinvels, A. and Hibert, M. (1992) Modelling of G-protein-coupled receptors: application to dopamine, adrenaline, serotonin, acetylcholine, and mammalian opsin receptors. J Med Chem. 18, 3448-3462.

Tse, A. and Hille, B. (1992) GnRH-induced Ca^{2+} oscillations and rhythmic hyperpolarizations of pituitary gonadotropes. Science 255, 462-464.

Tse, A., Tse, F.W., Almers, W. and Hille, B. (1993) Rhythmic exocytosis stimulated by GnRH-induced calcium oscillations in rat gonadotropes. Science 260, 82-84.

Tseng, M-J., Detjen, K., Struk, V. and Logsdon, C.D. (1995) Carboxyl-terminal domains determine internalization and recycling of bombesin receptor chimeras. J. Biol. Chem. 270, 18858-18864.

Tsuga, H., Kameyama, K., Haga, T., Kurose, H. and Nagao, T. (1994) Sequestration of muscarinic acetylcholine receptor m2 subtypes: facilitation by G protein-coupled receptor kinase (GRK2) and attenuation by a dominant-negative mutant of GRK2. J. Biol. Chem. 269, 32522-32527.

Tsuga, H., Kameyama, K., Haga, T., Honma, T., Lameh, J. and Sadee, W. (1998) Internalization and down-regulation of human muscarinic acetylcholine receptor m2 subtypes: role of third intracellular m2 loop and G protein-coupled receptor kinase 2. J. Biol. Chem. 273, 5323-5330.

Tsutsumi, M., Zhou, W., Millar, R.P., Mellon, P.L., Roberts, J.L., Flanagan, C.A., Dong, K., Gillo, B. and Sealfon, S.C. (1992) Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Mol. Endocrinol. 6, 1163-1169.

van der Merwe, PA., Millar, RP., Wakefield, IK., Davidson, JS. (1989) Mechanisms of luteinizing-hormone exocytosis in Staphylococcus aureus-α-toxin-permeabilized sheep gonadotropes. Biochem. J. 264, 901-908.

van der Sluijs, P., Hull, M., Zahraoui, A, Tavitian, A, Goud, B. and Mellman, I.S. (1992) The small GTP-binding protein rab4 controls an early sorting event on the endocytic pathway. Cell 70, 729-740.

van Koppen, C.J., Sell, A., Lenz, W. and Jakobs, K.H. (1994) Deletion analysis of the m4 muscarinic acetylcholine receptor: molecular determinants for activation of but not coupling to the G_i guanine-nucleotide-binding regulatory protein regulate receptor internalization. Eur. J. Biochem. 222, 525-531.

van Koppen, C.J., Lenz, W., Nunes, J.P.L., Zhang, C., Schmidt, M. and Jakobs, K.H. (1995) A role of memebrane proximal threonine residues conserved amaong guanine-nucleotide-binding-protein-coupled receptors in internalization of the m4 muscarinic acetylcholine receptor. Eur. J. Biochem. 234, 536-541.

Vögler, O., Bogatkewitsch, G.S., Wriske, C., Krummenerl, P., Jacobs, K.H. and van Koppen, C.J. (1998) Receptor subtype-specific regulation of muscarinic acetylcholine receptor sequestration by dynamin: distinct sequestration of m2 receptors. J. Biol. Chem. 273, 12155-12160.

Von Zastrow, M. and Kobilka, B.K. (1992) Ligand-regulated internalization and recycling of human β_2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. J. Biol. Chem. 267, 3530-3538.

Von Zastrow, M. and Kobilka, B.K. (1994) Antagonist-dependent and -independent steps in the mechanisms of adrenergic receptors internalization. J. Biol. Chem. 269, 18448-18452.

Waldo, G.L., Northup, J.K., Perkins, J.P. and Harden, T.K. (1983) Characterization of an altered membrane form of the β-adrenergic receptor produced during agonist-induced internalization. J. Biol. Chem. 258, 13900-13908.

Wang, J., Zheng, J., Anderson, J.L. and Toews, M.L. (1997) A mutation in the α_{1B}-adrenergic receptor that differentiates two steps in the pathway of receptor internalization. Mol. Pharmacol. 52, 306-313.

Wang, L.H., Südhof, T.C. and Anderson, R.G. (1995) The appendage domain of α-adaptin is a high-affinity binding site for dynamin. J. Biol. Chem. 270, 10079-10083.

Wang, Z., Wang, H. and Ascoli, M. (1993) Mutation of a highly conserved acidic residue present in the second intracellular loop of G protein-coupled receptors does not impair hormone binding or signal transduction of the luteinizing hormone/chorionic gonadotropin receptor. Mol. Endocrinol. 7, 85-93.

Wang, Z., Liu, X. and Ascoli, M. (1997) Phosphorylation of the lutropin/choriogonadotropin receptor facilitates uncoupling of the receptor from adenylyl cyclase and endocytosis of the bound hormone. Mol. Endocrinol. 11, 183-192.

Weiland, G.A., Minnemann, K.P. and Molinoff, P.B. (1979) Fundamental differences between the molecular interactions of agonists and antagonists with the β-adrenergic receptor. Nature (London) 281, 114-117.

Widmann, C., Dolci, W. and Thorens, B. (1996) Heterologous desensitization of the GLP-1 receptor by phorbol esters requires phosphorylation of the cytoplasmic tail at four different sites. J. Biol. Chem. 271, 19957-19963.

Widmann, C., Dolci, W. and Thorens, B. (1997) Internalization and homologous desensitization of the GLP-1 receptor depend on phosphorylation of the receptor carboxyl tail at the same three sites. Mol. Endocrinol. 11, 1094-1102.

Willars, G.B., McArdle, C.A. and Nahorski, S.R. (1998) Acute desensitization of phospholipase C-coupled muscarinic M3 receptors but not gonadotropin-releasing hormone receptors co-expressed in αT3-1 cells: implcations for mechanisms of rapid desensitization. Biochem. J. 333, 301-308.

Williams, B.Y., Dion, S.B. and Schonbrunn, A. (1998) Role of receptor and protein kinase C activation in the internalization of the gastrin-releasing hormone receptor. Mol. Pharmacol. 54, 889-898.

Windle, J.J., Weiner, R.I. and Mellon, P.L. (1990) Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol. Endocrinol. 4, 597-603.

Wynn, P.C., Suarez-Quian, C.A., Childs, G.V. and Catt, K.J. (1986) Pituitary binding and internalization of rdioiodinated gonadotropin-releasing hormone agonist and antagonist ligands in vitro and in vivo. Endocrinology, 119, 1852-1863.

Yu, R. and Hinkle, P.M. (1998) Signal transduction, desensitization, and recovery of response to thyrotropin-releasing hormone after inhibition of receptor internalization. Mol. Endocrinol. 12, 737-749.

Yu, S.S., Lefkowitz, R.J. and Hausdorff, W.P. (1993) β-adrenergic receptor sequestration: a potential mechanism of receptor resensitization. J. Biol. Chem. 268, 337-341.

Zhang, J., Ferguson, S.S.G., Barak, L.S., Ménard, L. and Caron, M.G. (1996a) Dynamin and β-arrestin reveal distinct mechanisms for G protein-coupled receptor internalization. J. Biol. Chem. 271, 18302-18305.

Zhang, J., Ferguson, S.S.G., Barak, L.S., Bodduluri, S.R., Laporte, S.A., Law, P.Y. and Caron, M.G. (1998) Role for G protein-coupled receptor kinase in agonist-specific regulation of μ-opiod receptor responsiveness. Proc. Natl. Acad. Sci. U.S.A. 95, 7157-7162.

Zhang, M., Turnbaugh, D., Cofie, D., Dogan, S., Koshida, H., Fugate, R. and Kem, D.C. (1996b) Protein kinase C modulation of cardiomyocyte angiotensin II and vasopressin receptor desensitization. Hypertension 27, 269-275.

Zhou, W., Flanagan, C., Ballesteros, J.A., Konvicka, K., Davidson, J.S., Weinstein, H., Millar, R.P. and Sealfon, S.C. (1994) A reciprocal mutation supports helix 2 and helix 7 proximity in the gonadotropin-releasing hormone receptor. Mol. Pharmacol. 45, 165-170.

Zhu, X., Gudermann, T., Birnbaumer, M. and Birnbaumer, L. (1993) A luteinizing hormone receptor with a severely truncated cytoplasmic tail (LHR-ct628)

desensitizes to the same degree as the full-length receptor. J. Biol. Chem. 268, 1723-1728.

Zhu, Z.Z., Wang, S. Z., Hu, J. and El-Fakahany, E.E. (1994) An arginine residue conserved in most G protein-coupled receptors is essential for the function of the m1 Muscarinic receptor. Mol. Pharmacol. 45, 517-523.

7 FIGURES

Figure 1. Possible heterologous desensitization pathways for GPCRs

Schematic representation of possible GPCR heterologous desensitization pathways. PLC, phospholipase C; DAG, diacylglycerol; PKA, protein kinase A; PKC, protein kinase C; CaM-dep kinase, Ca^{2+}-calmodulin-dependent kinase; cAMP, cyclic adenosine monophosphate; AC, adenylyl cyclase; G_q and G_s, heterotrimeric G proteins.

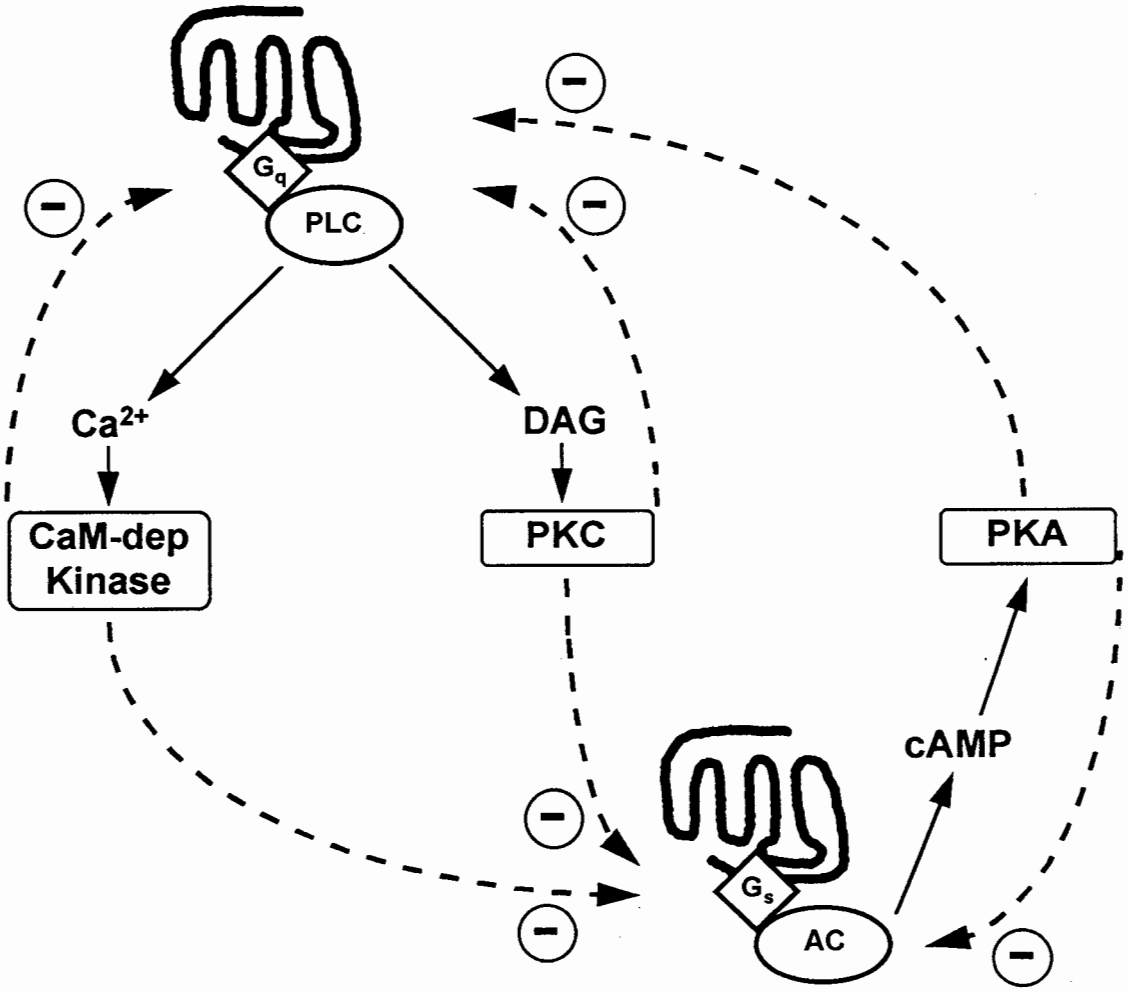


Figure 2. Possible relationships between agonist-promoted internalization of GPCRs and receptor activation, receptor-G protein coupling, and second messenger production

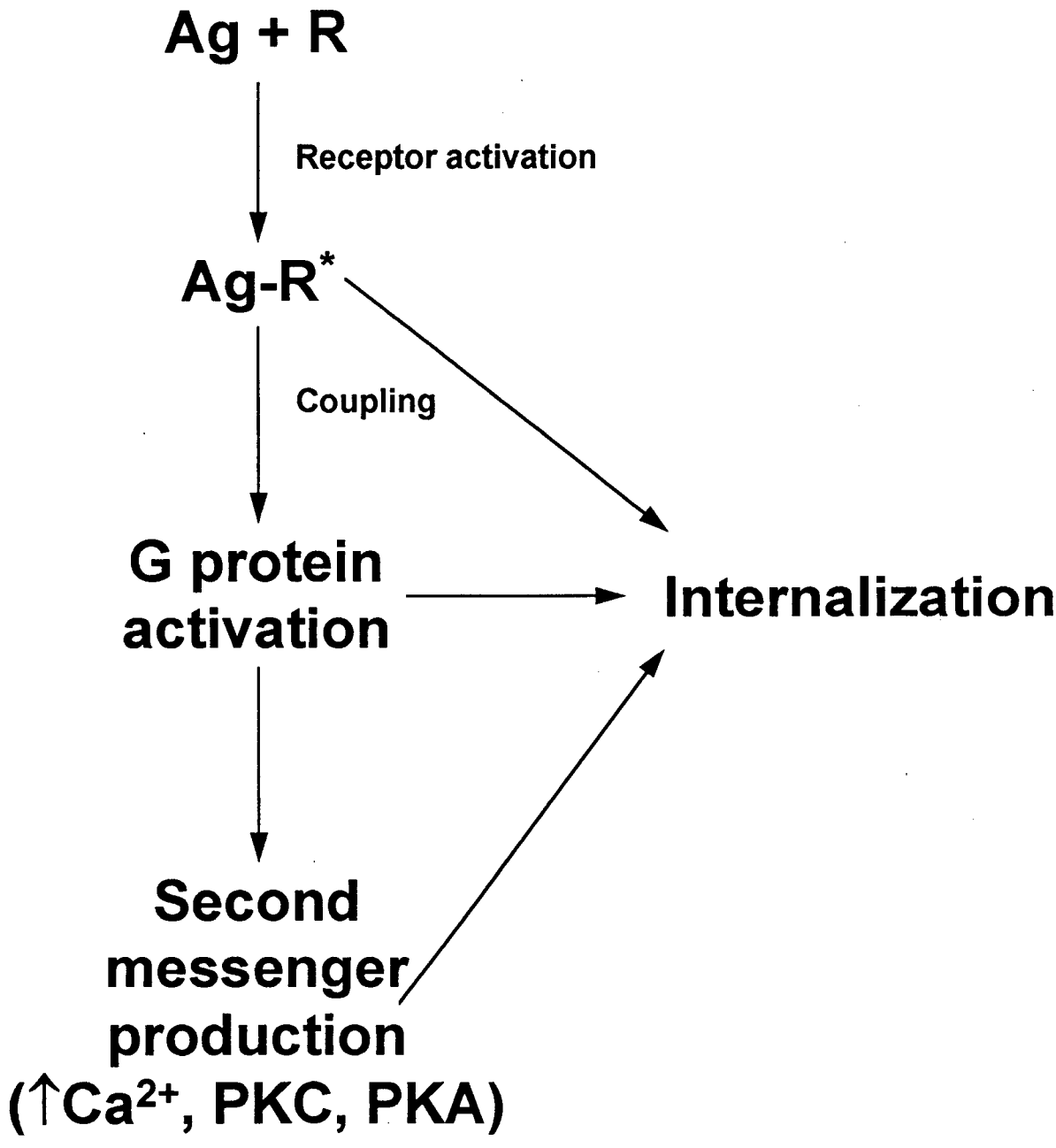
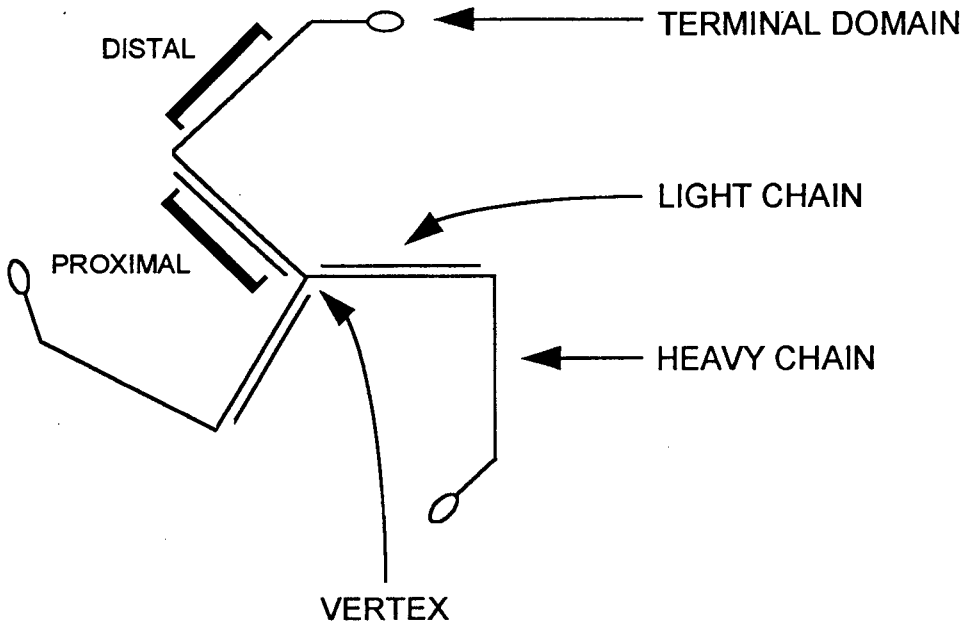


Figure 3. The clathrin triskelion and clathrin coat

Schematic drawing showing (a) the modular structure of the triskelion, and (b) the clathrin coat in relation to the budding vesicle.

(a)



(b)

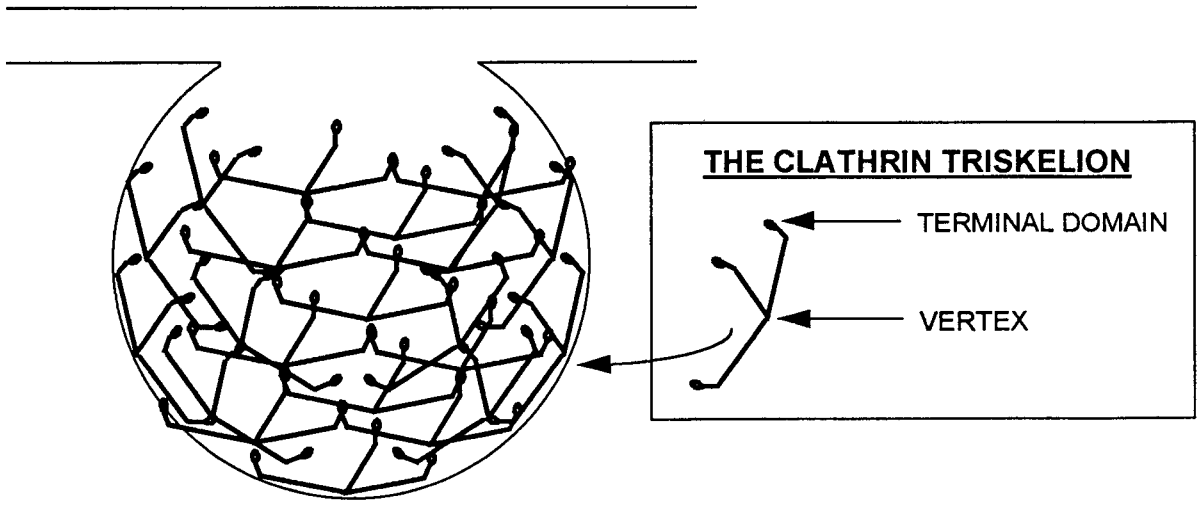


Figure 4. Regulation of GPCR responsiveness

Schematic representation of GPCR desensitization, agonist-promoted internalization, and resensitization following agonist activation. E, effector enzyme; α, β, and γ, subunits of heterotrimeric G protein; ☆, agonist; GRK, G protein-coupled receptor kinase; βarr, β-arrestin; P, phosphate; ◆, dynamin; - - -, clathrin coat. See text for details. (modified from Ferguson et al., 1996b).

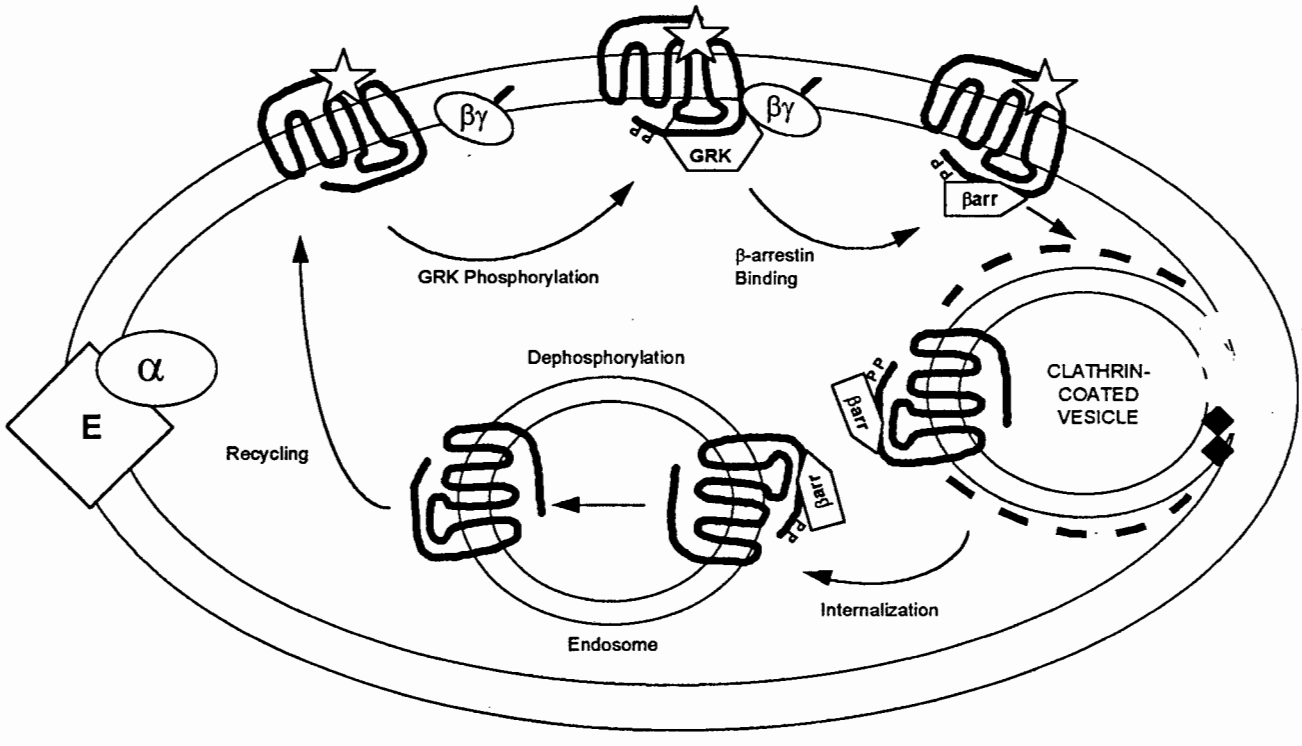


Figure 5. Amino acid sequence of the mouse GnRH receptor

The residues mutated or investigated in this thesis are shown as filled circles. Other numbered residues are discussed in the text. Branched lines represent carbohydrate moieties, and short solid lines represent disulphide bridges.

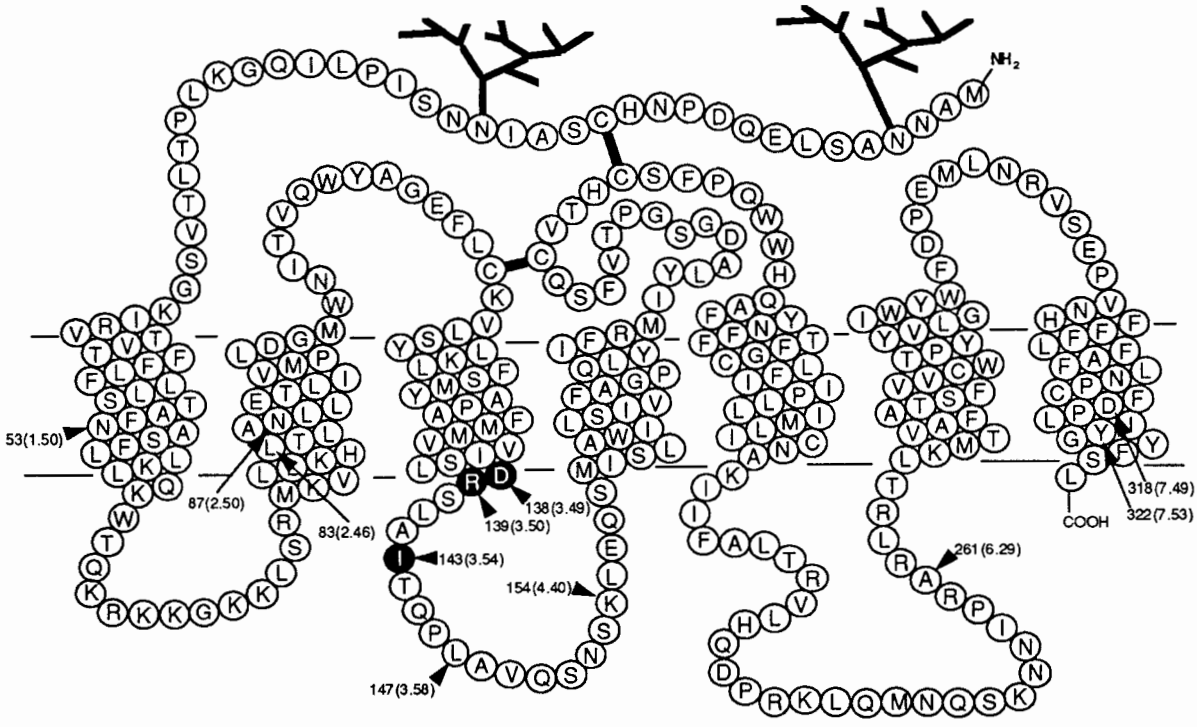


Figure 6. Amino acid sequence of the chicken GnRH receptor

The residues mutated in this thesis are shown as filled circles. The short solid line represents a disulphide bridge. Truncations of the cytoplasmic tail are indicated by short lines.

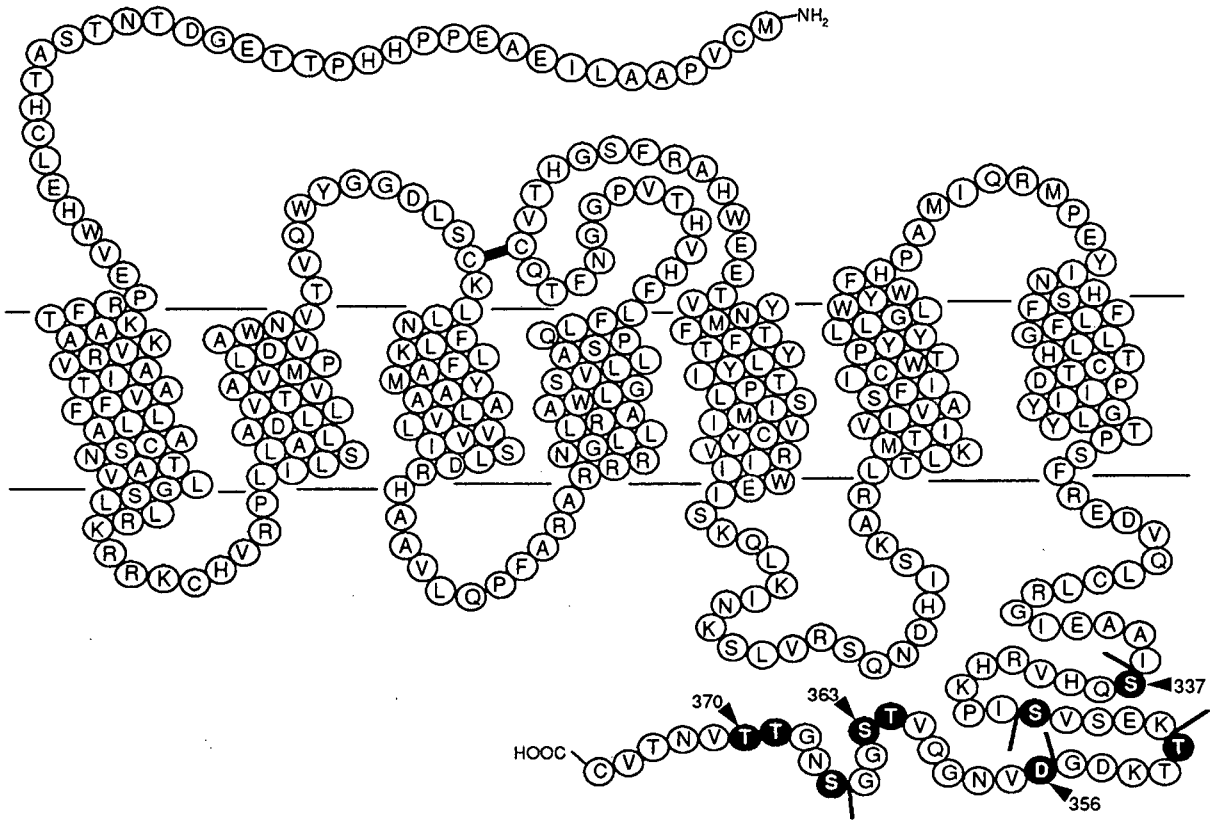


Figure 7. Iodination and HPLC purification of [D-Ala^6]GnRH

Iodination was performed as described in **Section 2.5**. The iodination shown is typical of three iodinations. The first peak represents free ^{125}I followed by the peptide (^{125}I-[D-Ala^6]GnRH) peak at fraction 35 (*).

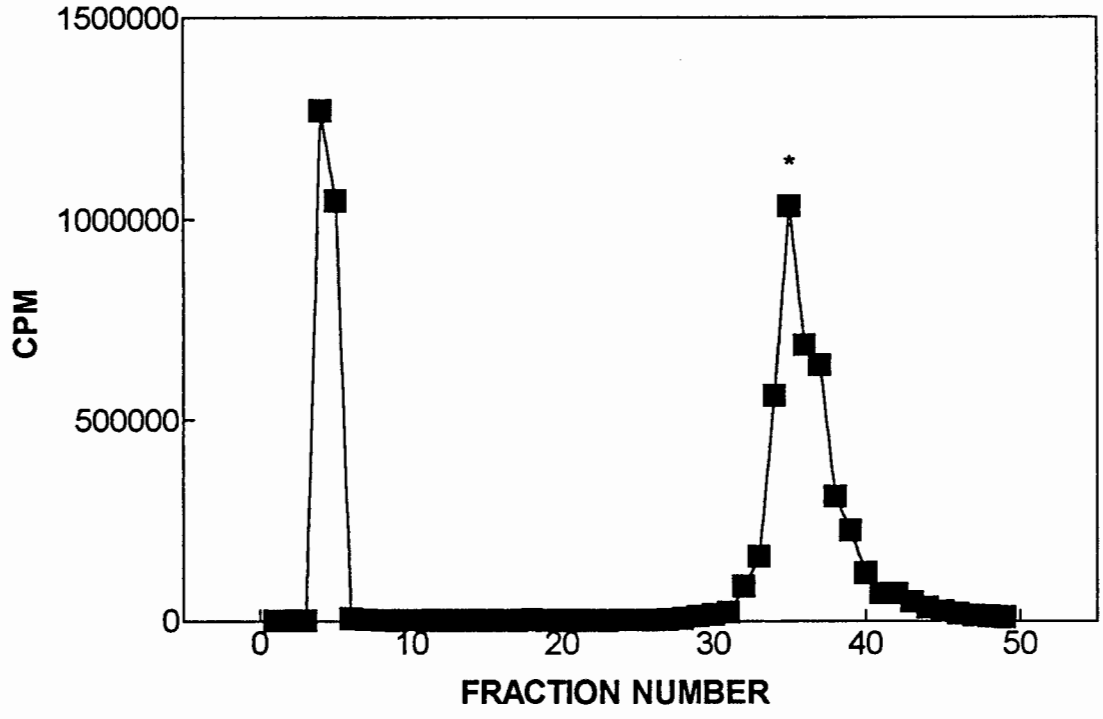


Figure 8. Iodination and HPLC purification of Antagonist 26

Iodination was performed as described in **Section 2.5**. The iodination shown is typical of four iodinations. The first peak represents free ^{125}I followed by the peptide (^{125}I-Antagonist 26) peak at fraction 42 (*).

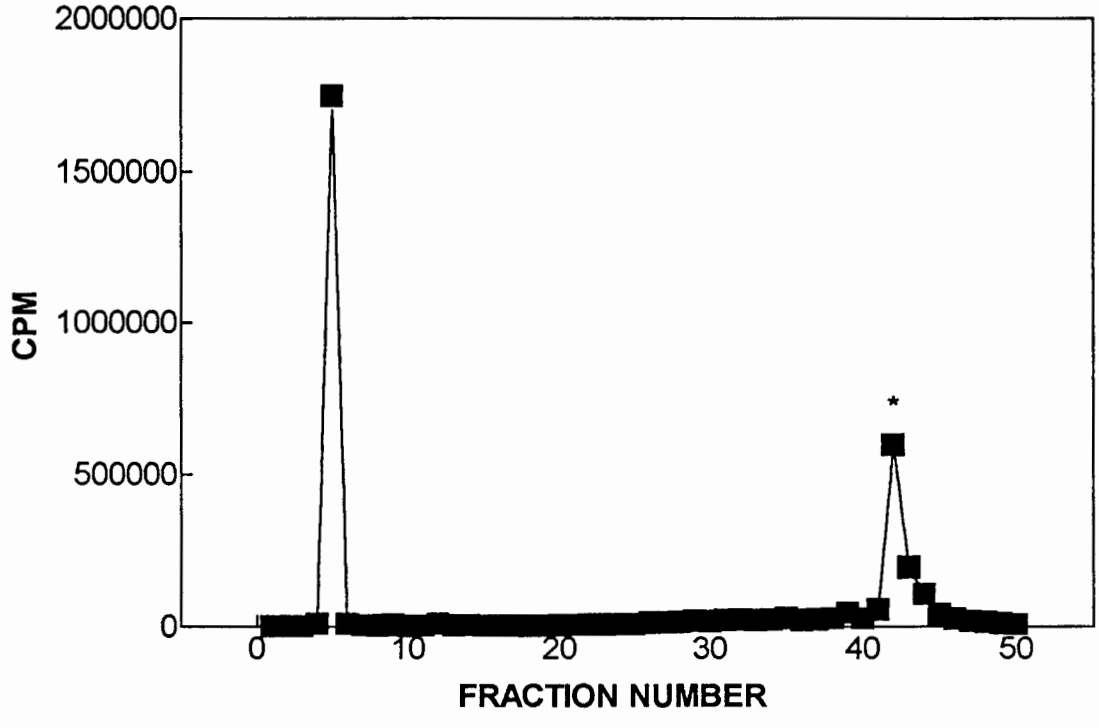


Figure 9. Iodination and HPLC purification of [His^5,D-Tyr^6]GnRH

Iodination was performed as described in **Section 2.5**. The iodination shown is typical of four iodinations. The first peak represents free ^{125}I followed by the peptide (^{125}I-[His^5, D-Tyr^6]GnRH) peak at fraction 31 (*). The identity of the material eluting in the middle peak is not known.

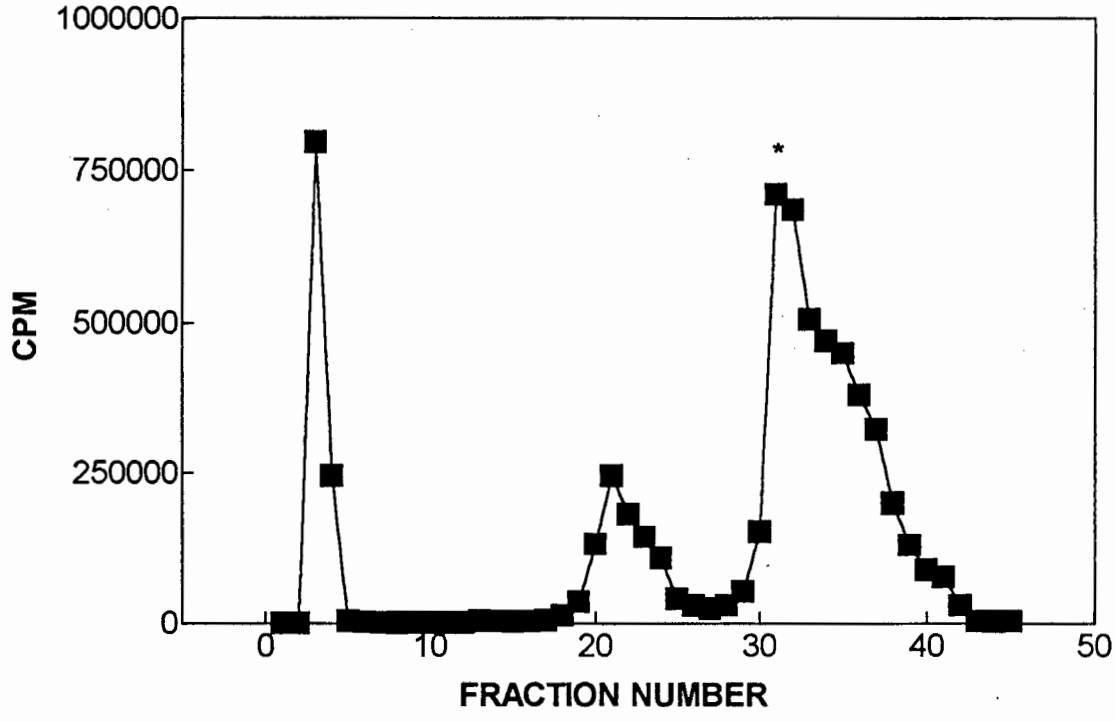


Figure 10. GnRH agonist binding displaced by GnRH (heterologous displacement) in WT, D138N and R139Q mouse GnRHRs

Displacement curves of the binding of ^{125}I-[D-Ala^{6}]GnRH (agonist) in the presence of increasing concentrations of unlabeled mGnRH to COS-1 cells expressing mWT ( ), mD138N (O), and mR139Q (Δ) GnRHRs. B_o refers to radioligand bound in the absence of competing unlabeled ligand. Non-specific binding (NSB) determined in the presence of 100 nM unlabeled Antagonist 26 has been subtracted from the data. The experiment shown is representative of three independent experiments performed in triplicate.

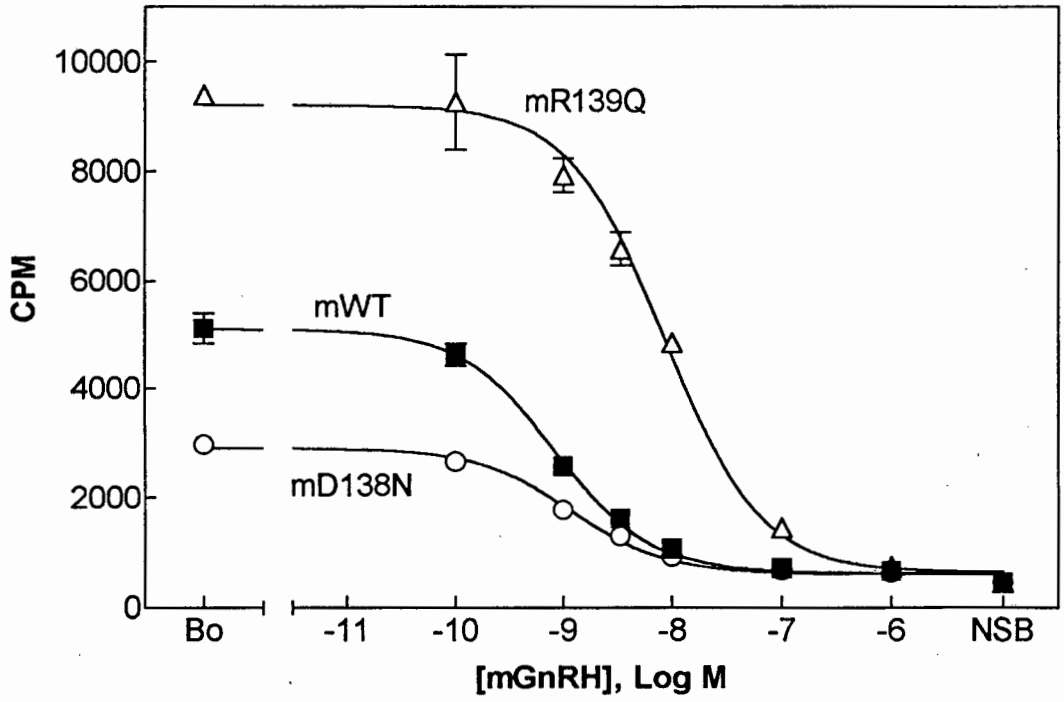


Figure 11. GnRH agonist binding displaced by GnRH agonist (homologous displacement) in WT, D138N, and R139Q mouse GnRHRs

Displacement curves of the binding of ^{125}I-[D-Ala^6]GnRH (agonist) in the presence of increasing concentrations of unlabeled [D-Ala^6]GnRH to COS-1 cells expressing mWT (■), mD138N (O), and mR139Q (Δ) GnRHRs. B_o refers to radioligand bound in the absence of competing unlabeled ligand. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each dilution. The experiment shown is representative of three independent experiments performed in triplicate.

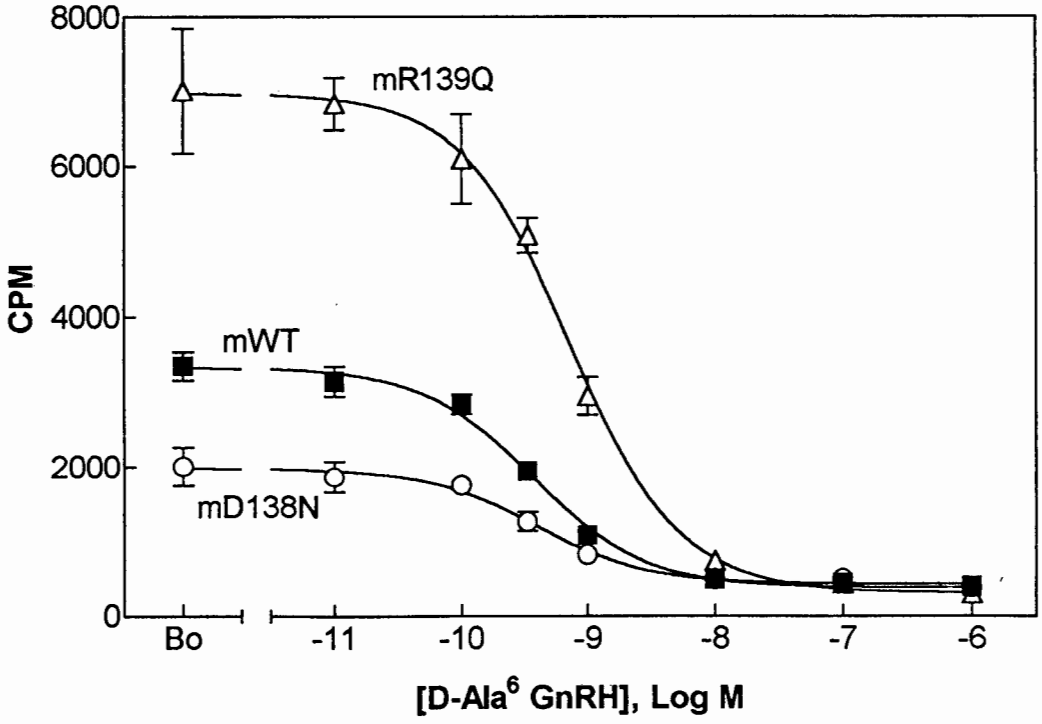


Figure 12. GnRH antagonist binding displaced by antagonist (homologous displacement) in WT, D138N, and R139Q mouse GnRHRs

Displacement curves of the binding of ^{125}I-Antagonist 26 in the presence of increasing concentrations of unlabeled Antagonist 26 to COS-1 cells expressing mWT (■), mD138N (O), and mR139Q (Δ) GnRHRs. B_o refers to radioligand bound in the absence of competing unlabeled ligand. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each dilution. The experiment shown is representative of three independent experiments performed in triplicate.

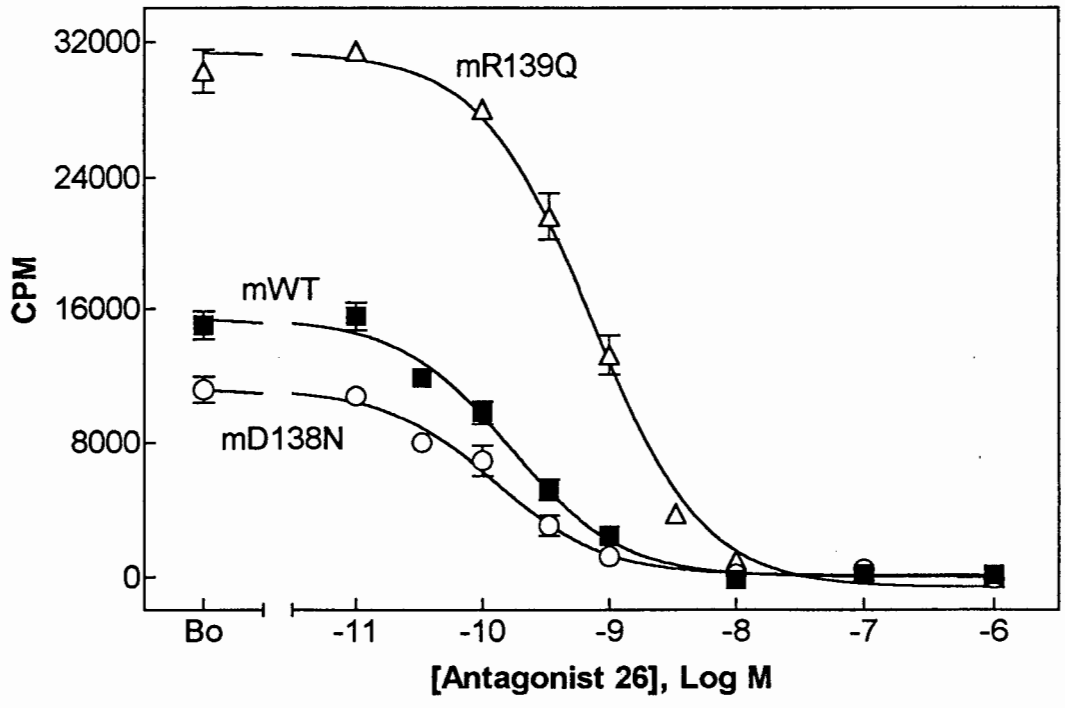


Figure 13. Agonist-stimulated inositol phosphate production mediated by WT, D138N, and R139Q mouse GnRHRs

The curves show total ^{3}H-IP production in the presence of Li^{+} in response to stimulation with GnRH for 1 hour at 37°C in COS-1 cells pre-labeled with [^{3}H]-myo-inositol and expressing mWT (■), mD138N (O), and mR139Q (Δ) GnRHRs. The experiment shown is representative of three independent experiments performed in duplicate.

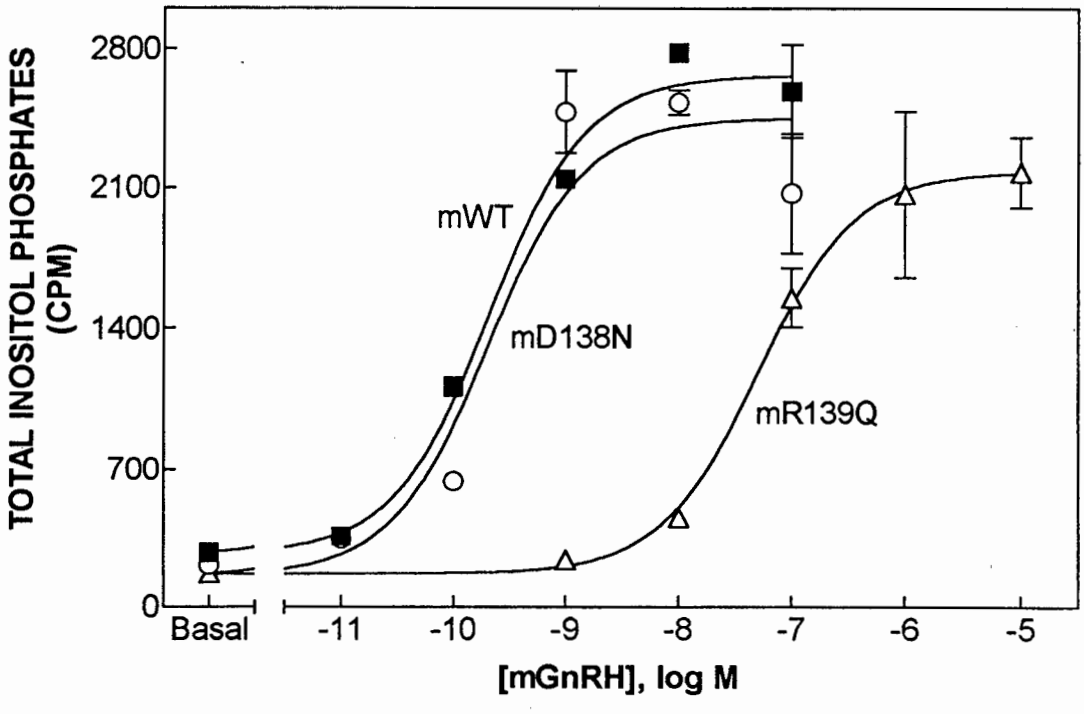


Figure 14. GnRH agonist binding by WT, D138A, R139K, and R139H mouse GnRHRs

The binding of ^{125}I-[D-Ala^6]GnRH (agonist) to COS-1 cells expressing mWT, mD138A, mR139K, and mR139H GnRHRs. B_o refers to radioligand bound in the absence of competing unlabeled ligand. Non-specific binding (NSB) determined in the presence of 100 nM unlabeled Antagonist 26. The experiment shown is representative of two independent experiments performed in triplicate.

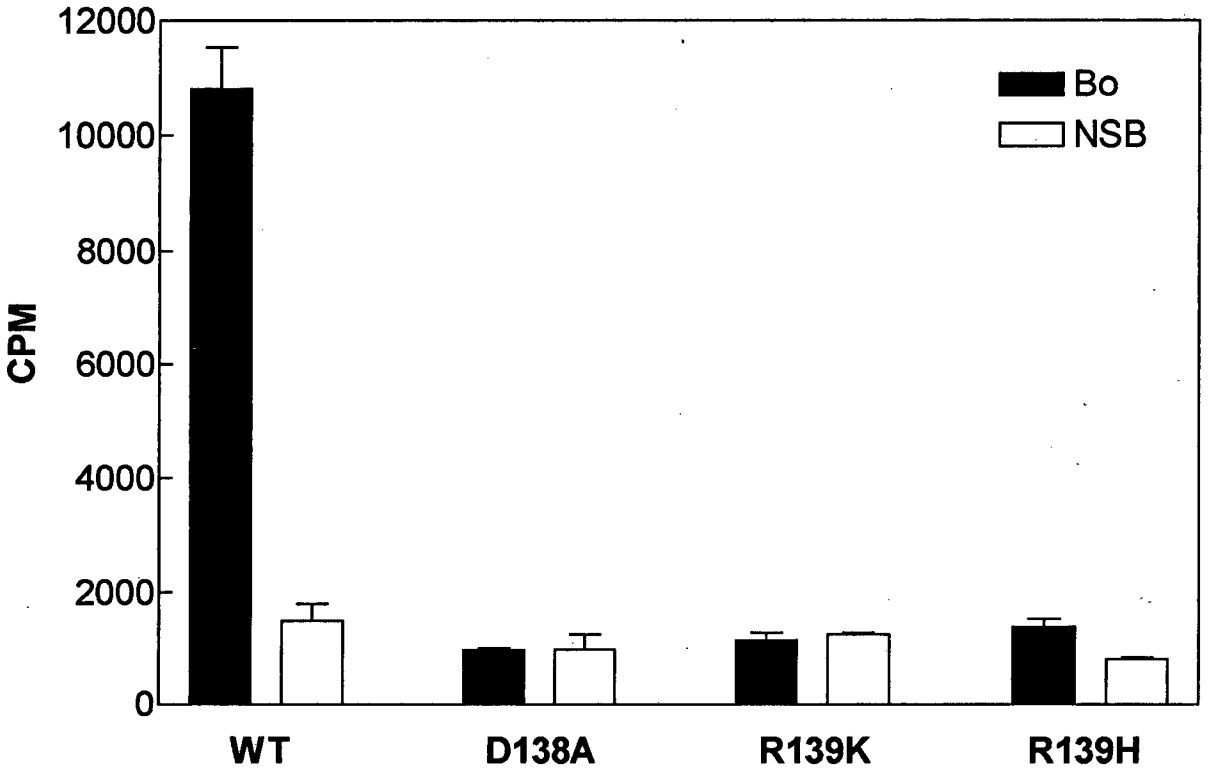


Figure 15. GnRH antagonist binding by WT, D138A, R139K, and R139H mouse GnRHRs

The binding of ^{125}I-Antagonist 26 to COS-1 cells expressing mWT, mD138A, mR139K, and mR139H GnRHRs. B_o refers to radioligand bound in the absence of competing unlabeled ligand. Non-specific binding (NSB) determined in the presence of 100 nM unlabeled Antagonist 26. Data points represent the mean ± S.E.M. of two independent experiments performed in triplicate.

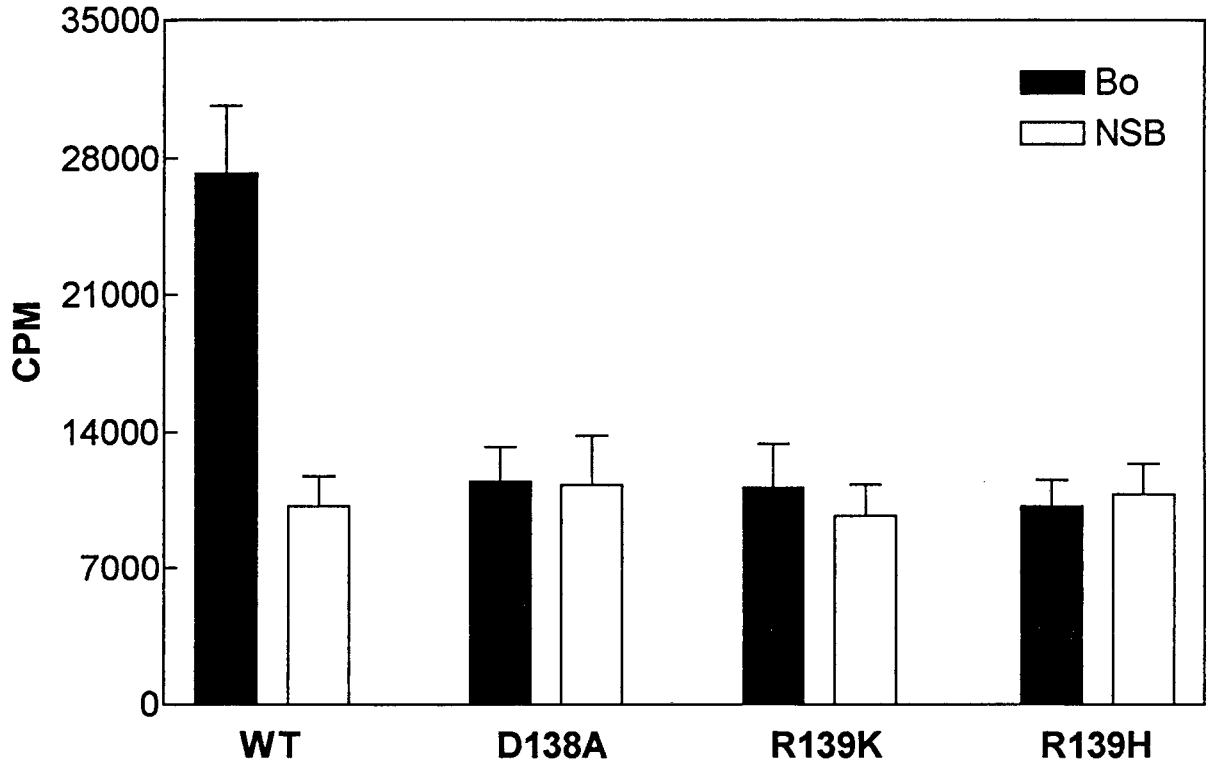


Figure 16. Agonist-stimulated inositol phosphate production mediated by WT, D138A, R139K, and R139Q mouse GnRHRs

Total ^{3}H-IP production in the presence of Li^{+} in response to stimulation with GnRH for 1 hour in COS-1 cells pre-labeled with [^{3}H]-myo-inositol and expressing mWT, mD138A, mR139K, and mR139H GnRHRs. The experiment shown is representative of two independent experiments performed in duplicate.

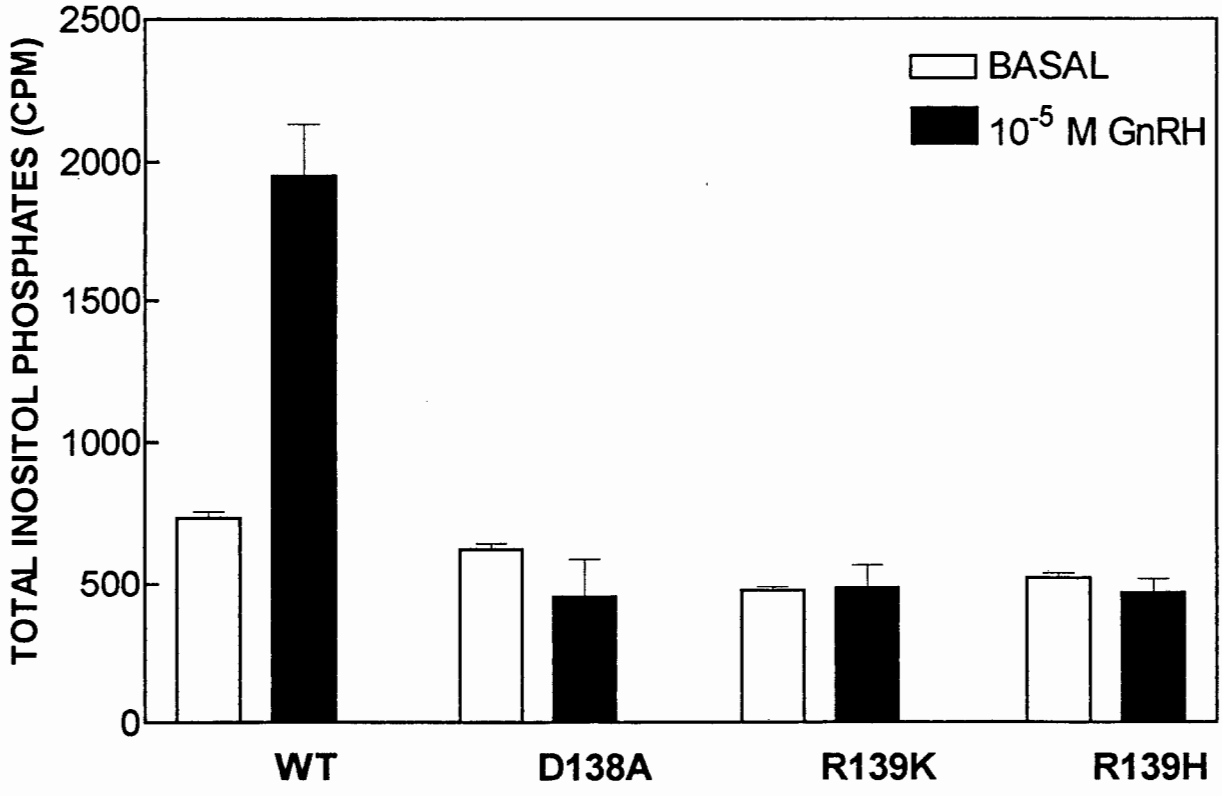


Figure 17. Temperature modulation of GnRH agonist binding to WT mouse GnRHRs

Intact αT3-1 cells in 35 mm culture dishes were allowed to bind ^{125}I-[D-Ala^6]GnRH at either 4°C first (□) or 37°C first (■), for various time-points, followed by a shift to the opposite temperature, for various time-points. At each time-point the cells were washed rapidly with ice-cold PBS, and then solubilized in 1 N NaOH to determine the total amount of radioligand bound. Non-specific binding determined in the presence of 100 nM unlabeled Antagonist 26 has been subtracted from the data. Data points represent the mean ± S.E.M. of two independent experiments performed in duplicate.

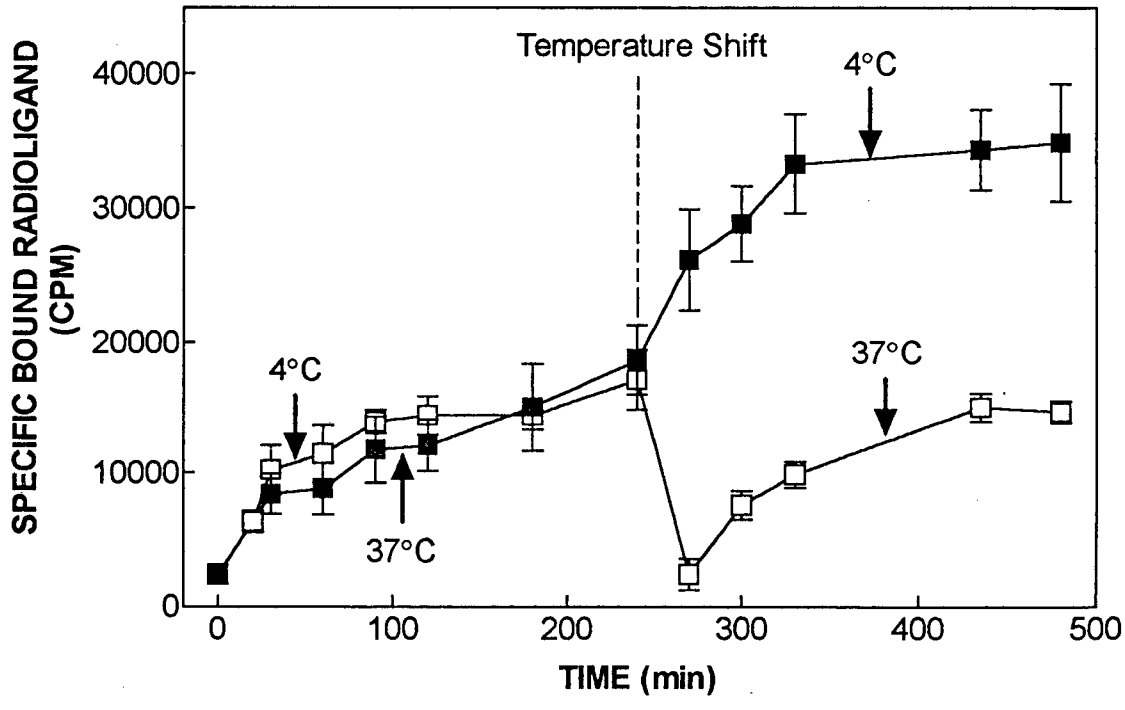


Figure 18. Temperature modulation of GnRH agonist binding to WT, D138N, and R139Q mouse GnRHRs

Intact COS-1 cells expressing mWT (■), mD138N (O), and mR139Q (Δ) GnRHRs were incubated with agonist radioligand ^{125}I-[D-Ala^6]GnRH for 3 hrs at 4°C, after which they were warmed to 37°C and incubated for the indicated times without changing the medium, then acid-washed to determine surface-bound radioligand. Cells shown at t = 0 were acid-washed without warming to 37°C. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each time-point. Data points represent the mean ± S.E.M. of three independent experiments performed in triplicate.

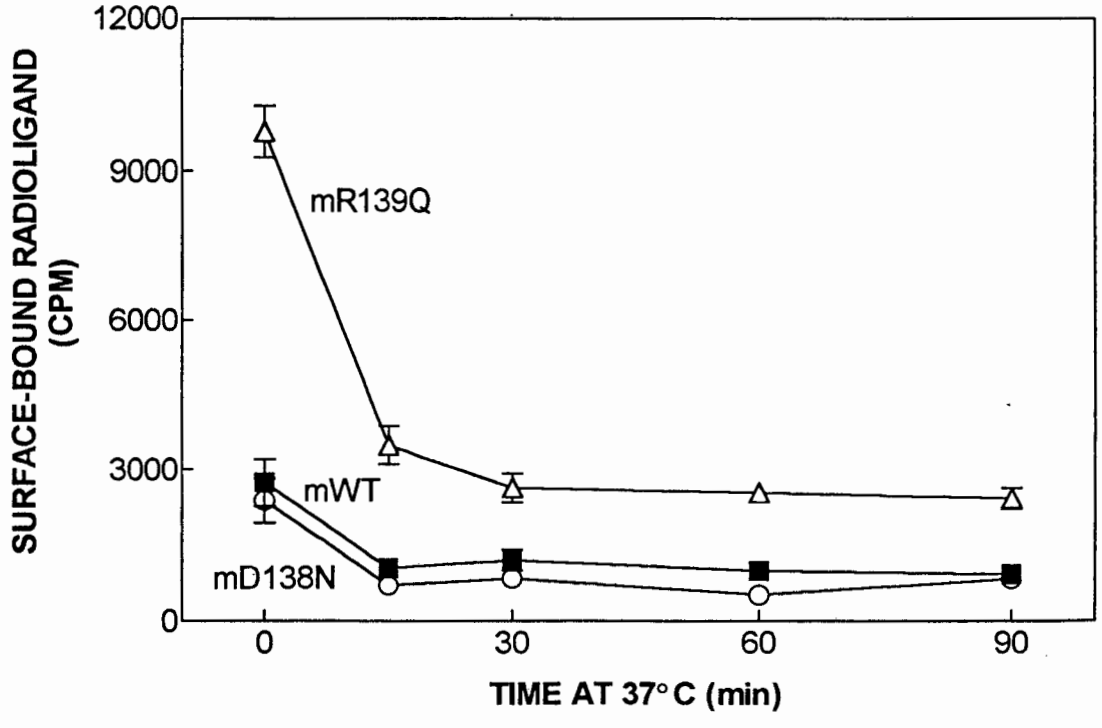


Figure 19. Time-course of binding of ^{125}I-[D-Ala^6]GnRH at 4°C

Intact αT3-1 cells which endogenously express mWT GnRHRs (■) were incubated with the agonist radioligand ^{125}I-[D-Ala^6]GnRH (10^5 cpm/well) for the times shown at 4°C. Cells were then washed, solubilized in 1 N NaOH and the total radioactivity counted in a γ-counter. Non-specific binding determined in the presence of 100 nM unlabeled Antagonist 26 has been subtracted from the data. The experiment shown is representative of two independent experiments performed in duplicate.

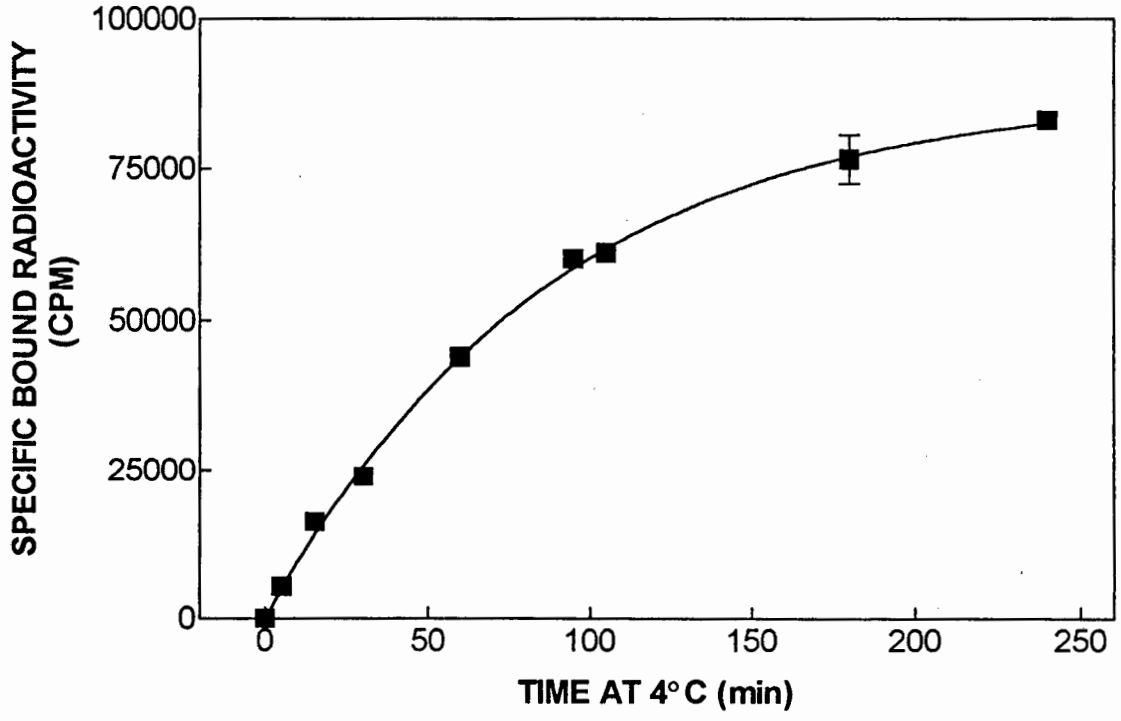


Figure 20. Optimization of acid-wash conditions for ^{125}I-[D-Ala^6]GnRH

Intact COS-1 cells expressing mWT GnRHRs were incubated with the agonist radioligand ^{125}I-[D-Ala^6]GnRH for 3 hrs at 0°C. Cells were washed twice rapidly with ice-cold PBS followed by the indicated wash (also ice-cold), which was collected. The cells were then solubilized in 1 N NaOH. The two fractions were counted in a γ-counter and the acid-sensitive radioactivity expressed. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted from the data. The experiment shown is representative of two independent experiments performed in duplicate.

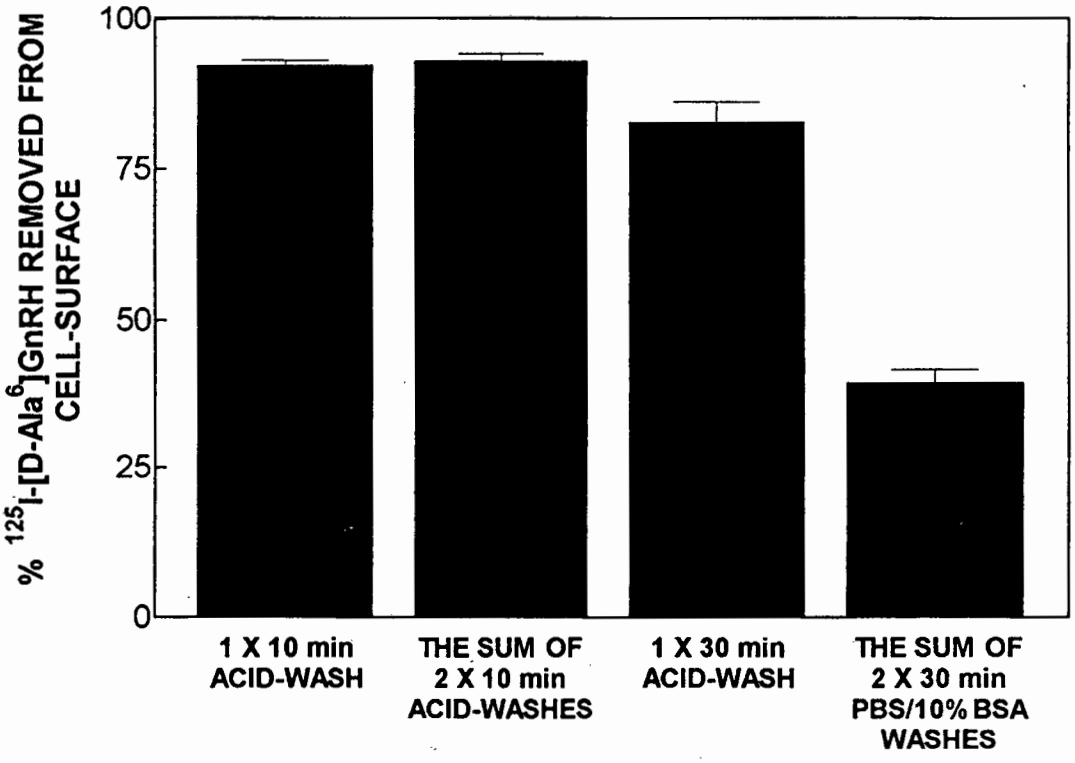


Figure 21. Time-course of binding of ^{125}I-Antagonist 26 at 4°C

Intact αT3-1 cells which endogenously express mWT GnRHRs (■) were incubated with the agonist radioligand ^{125}I-Antagonist 26 (10^5 cpm/well) for the times shown at 4°C. Cells were then washed, solubilized in 1 N NaOH and the total radioactivity counted in a γ-counter. Non-specific binding determined in the presence of 100 nM unlabeled Antagonist 26 has been subtracted from the data. The experiment shown is representative of a single experiment performed in duplicate.

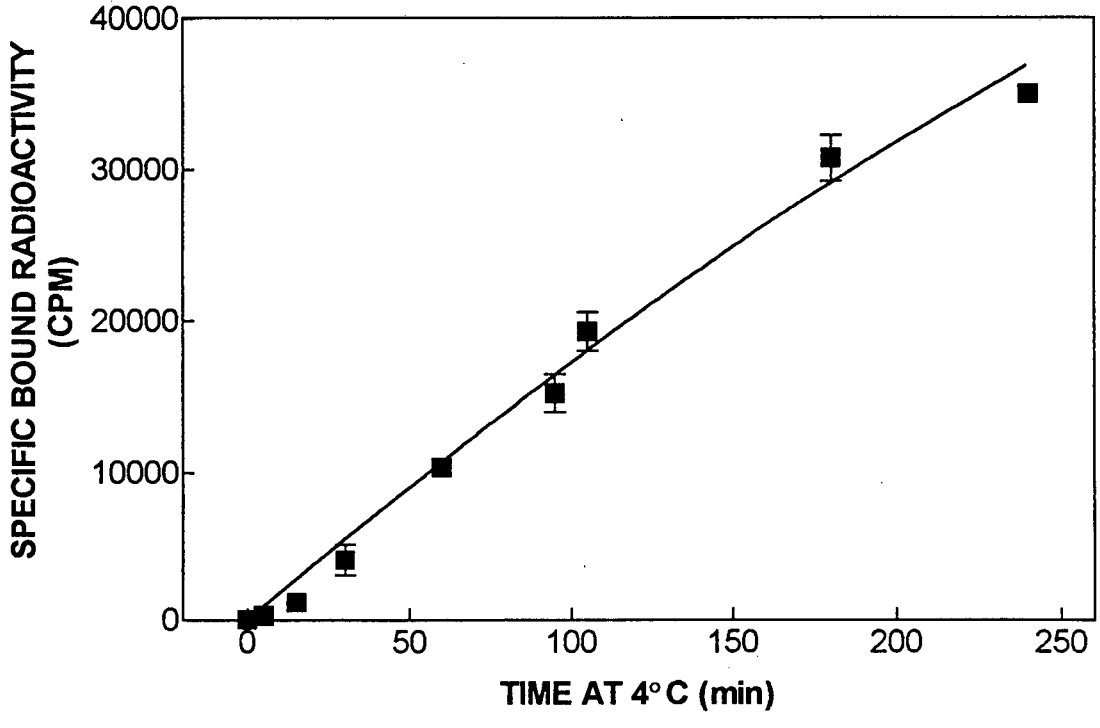


Figure 22. Optimization of acid-wash conditions for ^{125}I-Antagonist 26

Intact αT3-1 cells which endogenously express mWT GnRHRs were incubated with the antagonist radioligand ^{125}I-Antagonist 26 for 3 hrs at 0°C. Cells were washed twice rapidly with ice-cold PBS followed by the indicated wash (also ice-cold), which was collected. The cells were then solubilized in 1 N NaOH. The two fractions were counted in a γ-counter and the acid-sensitive radioactivity expressed. Non-specific binding determined in the presence of 100 nM unlabeled Antagonist 26 has been subtracted from the data. The experiment shown is representative of two independent experiments performed in duplicate.

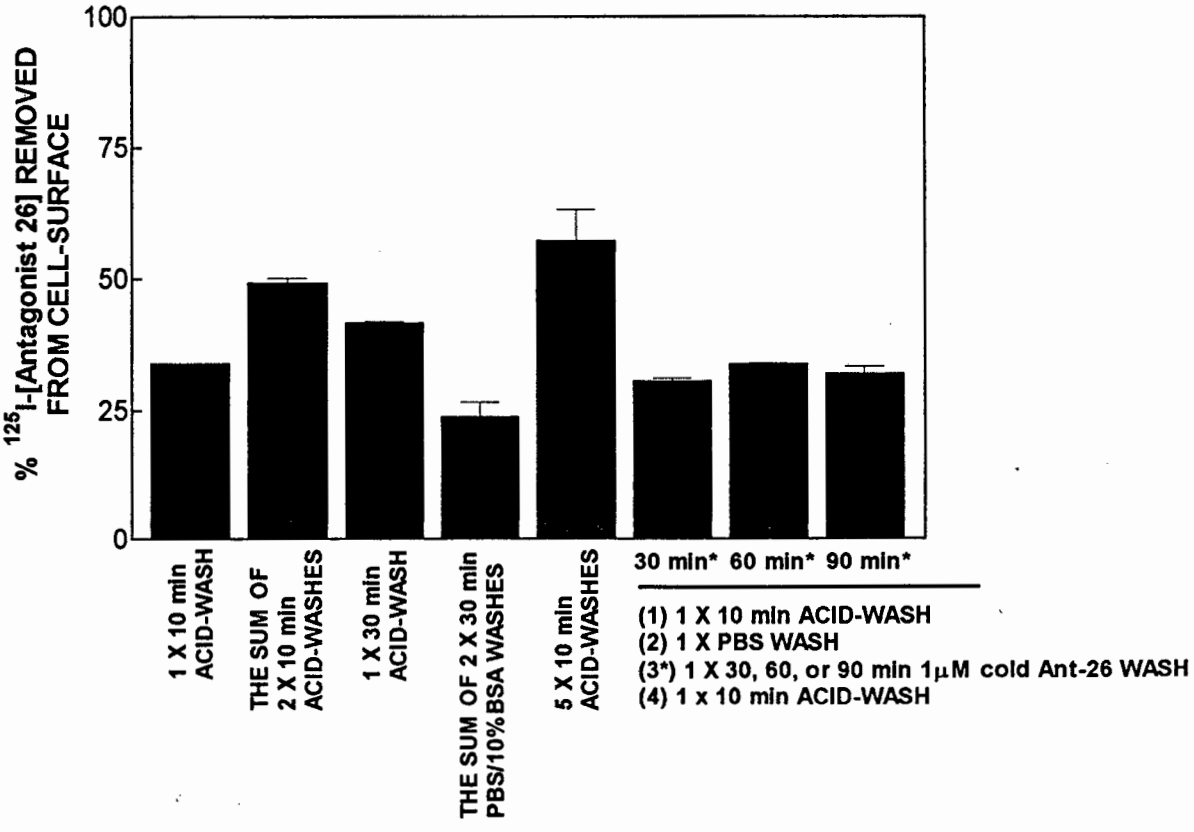


Figure 23. Comparison of internalization of agonist- and antagonist-occupied GnRH receptors (DAB cross-linking method)

Internalization of ^{125}I-Antagonist 26 (left panel) and ^{125}I-[D-Ala^{6}]GnRH (right panel) in COS-1 cells expressing mWT GnRHRs was determined by the DAB cross-linking method as described in **Section 2.7**. Internalized radioligand is expressed as % of total cell-associated radioligand. Non-specific binding determined in the presence of 100 nM unlabeled Antagonist 26 has been subtracted from the data. Data points are the mean ± S.E.M. of two independent experiments performed in triplicate.

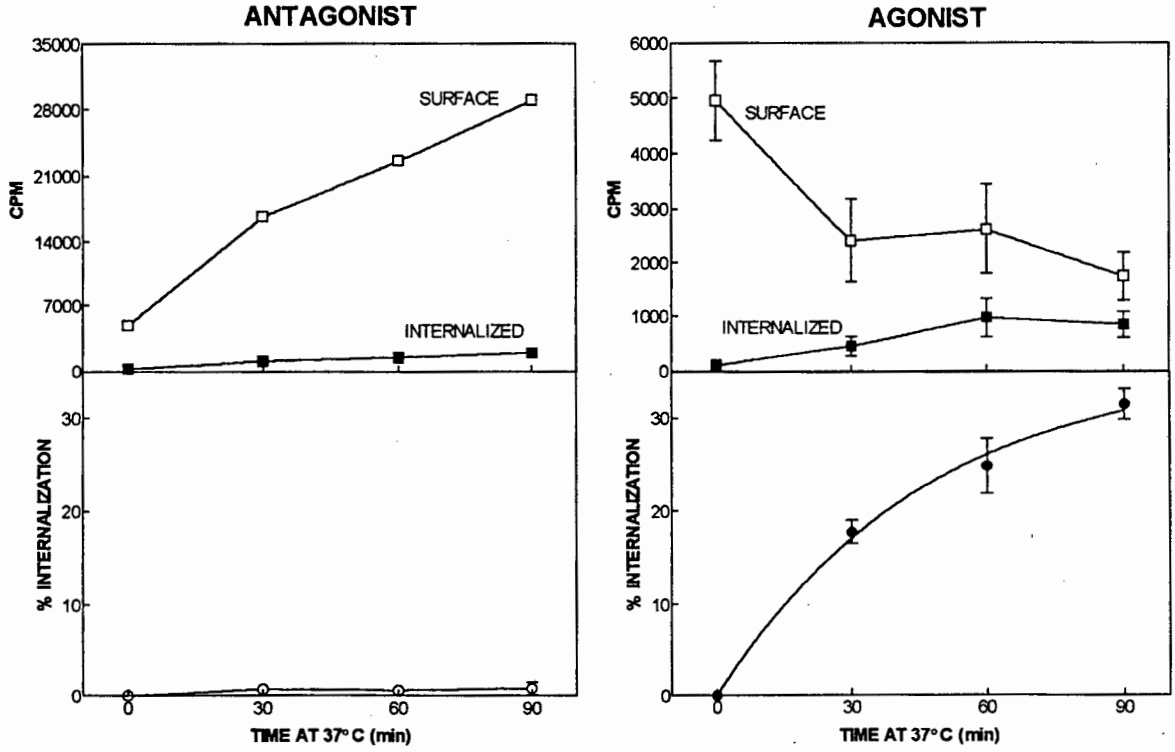


Figure 24. Acid-sensitive and -resistant ^{125}I-[D-Ala^6]GnRH fractions from untransfected COS-1 cells and COS-1 cells expressing WT mouse GnRHRs (acid-wash method)

Acid-sensitive and acid-resistant ^{125}I-[D-Ala^6]GnRH fractions from untransfected COS-1 cells (□) and COS-1 cells expressing mWT GnRHRs (■) were determined by the acid-wash method as described in **Section 2.7**. Data points are the mean ± S.E.M. of a single representative experiment performed in triplicate. This figure is included to demonstrate the non-specific components (□) in the acid-sensitive and acid-resistant fractions, determined in parallel using untransfected COS-1 cells.

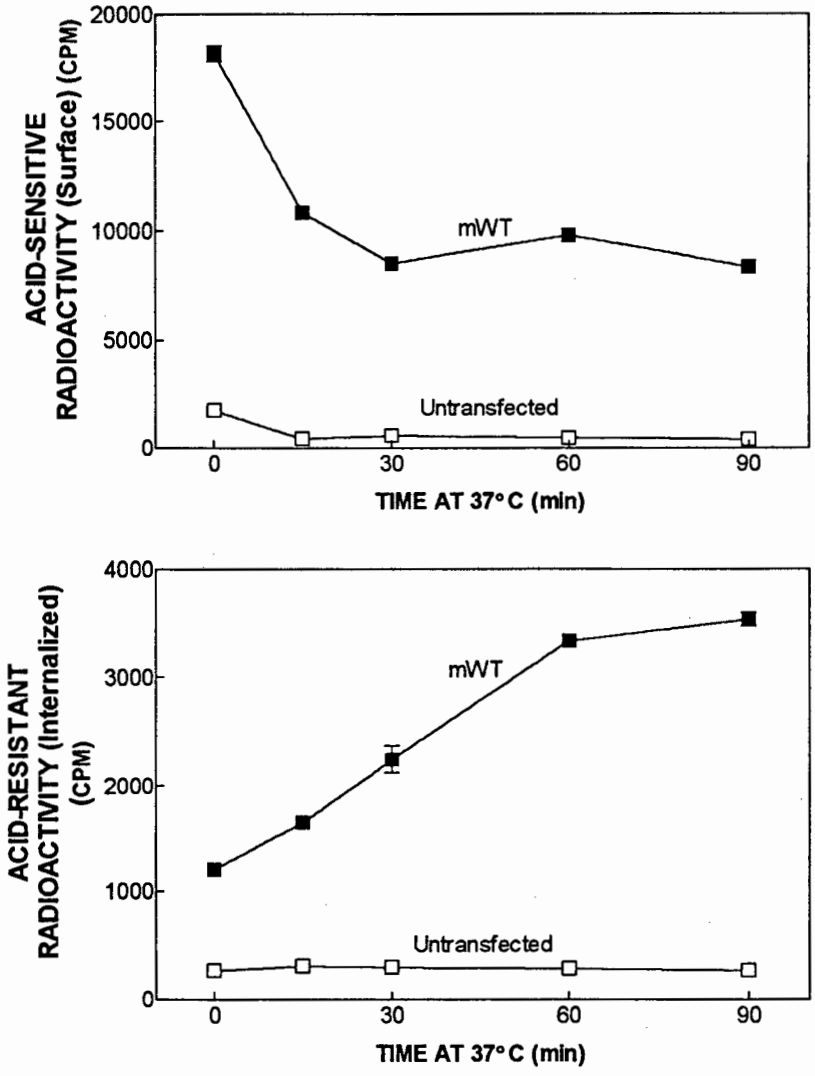


Figure 25. Internalization of ^{125}I-[D-Ala^6]GnRH mediated by WT, D138N, and R139Q mouse GnRHRs (acid-wash method)

Internalization of ^{125}I-[D-Ala^6]GnRH in COS-1 cells expressing mWT (■), mD138N (O), and mR139Q (Δ) GnRHRs was determined by the acid-wash method as described in **Section 2.7**. Internalized radioligand is expressed as % of total cell-associated radioligand. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each time-point. Data points are the mean ± S.E.M. of three independent experiments performed in triplicate.

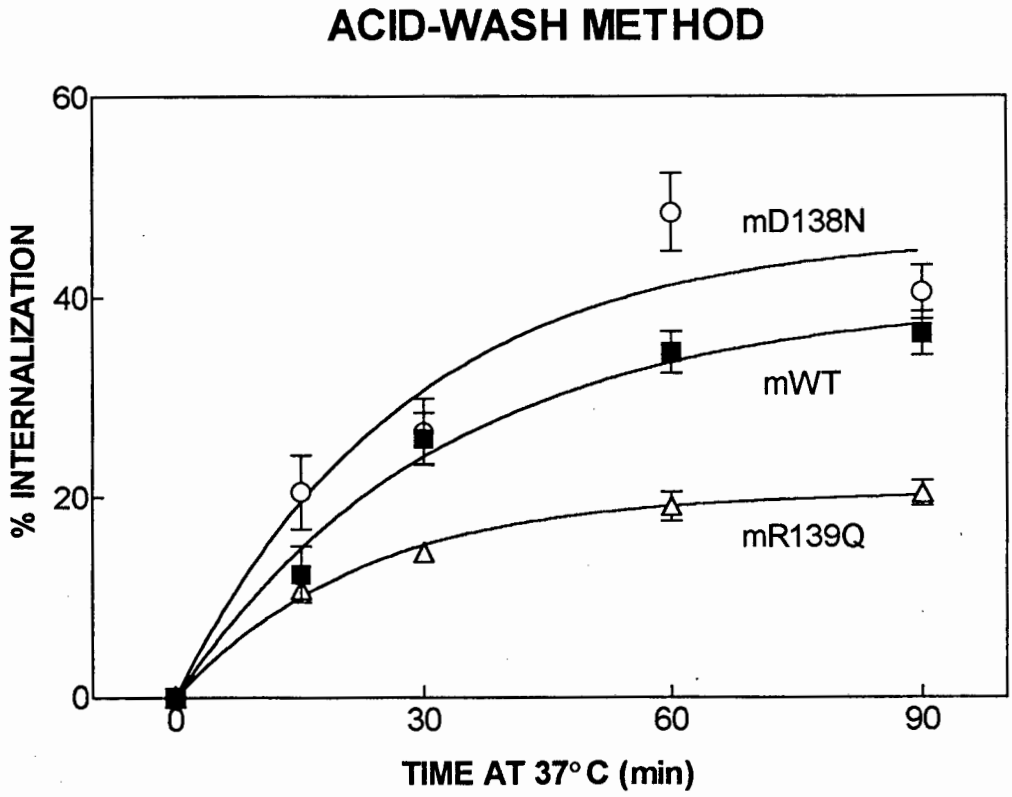


Figure 26. Internalization of ^{125}I-[D-Ala^6]GnRH mediated by WT, D138N, and R139Q mouse GnRHRs (DAB cross-linking method)

Internalization of ^{125}I-[D-Ala^6]GnRH in COS-1 cells expressing mWT (■), mD138N (○), and mR139Q (Δ) GnRHRs was determined by the DAB cross-linking method as described in **Section 2.7**. Internalized radioligand is expressed as % of total cell-associated radioligand. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each time-point. Data points are the mean ± S.E.M. of three independent experiments performed in triplicate.

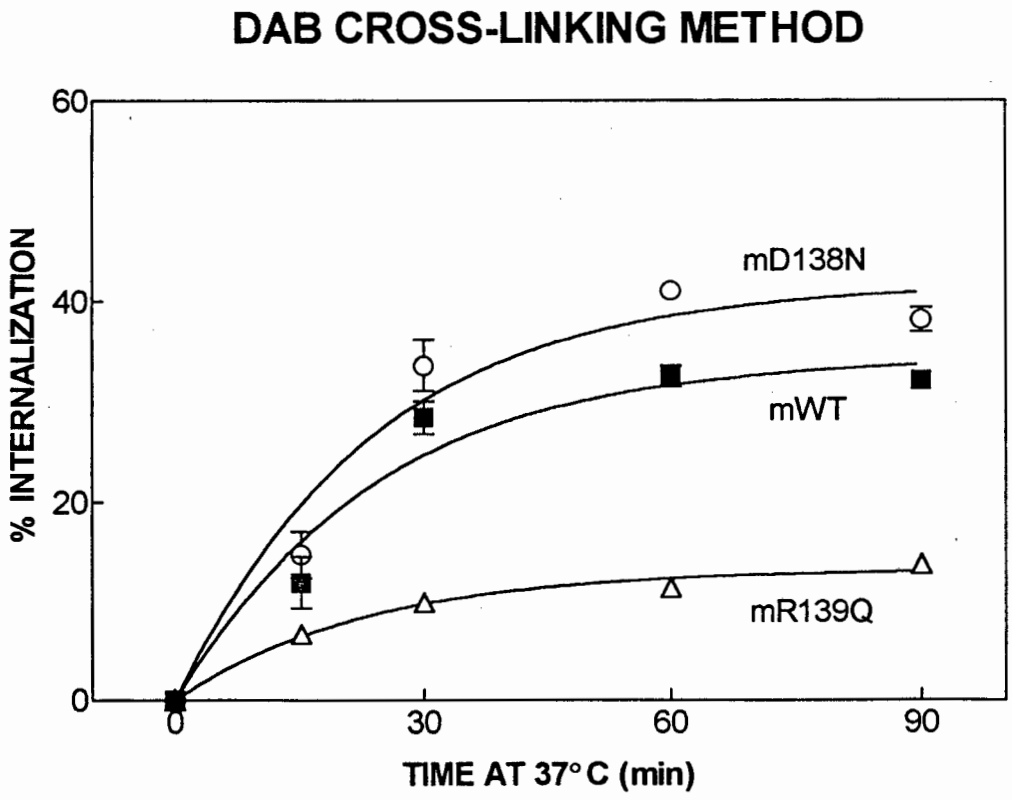


Figure 27. Effect of phorbol ester on agonist-promoted internalization of WT and R139Q mouse GnRHRs

Internalization of ^{125}I-[D-Ala^6]GnRH in COS-1 cells expressing mWT GnRHR in the absence (■) and presence (□) of PMA, and mR139Q GnRHR in the absence (▲) and presence (Δ) of PMA, was determined by the acid-wash method as described in **Section 2.7**. Cells were pre-incubated for 1 hr at 37°C with 100 nM phorbol 12-myristate 13-acetate before the addition of radioligand. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each time-point. Data points are the mean ± S.E.M. of two independent experiments performed in duplicate.

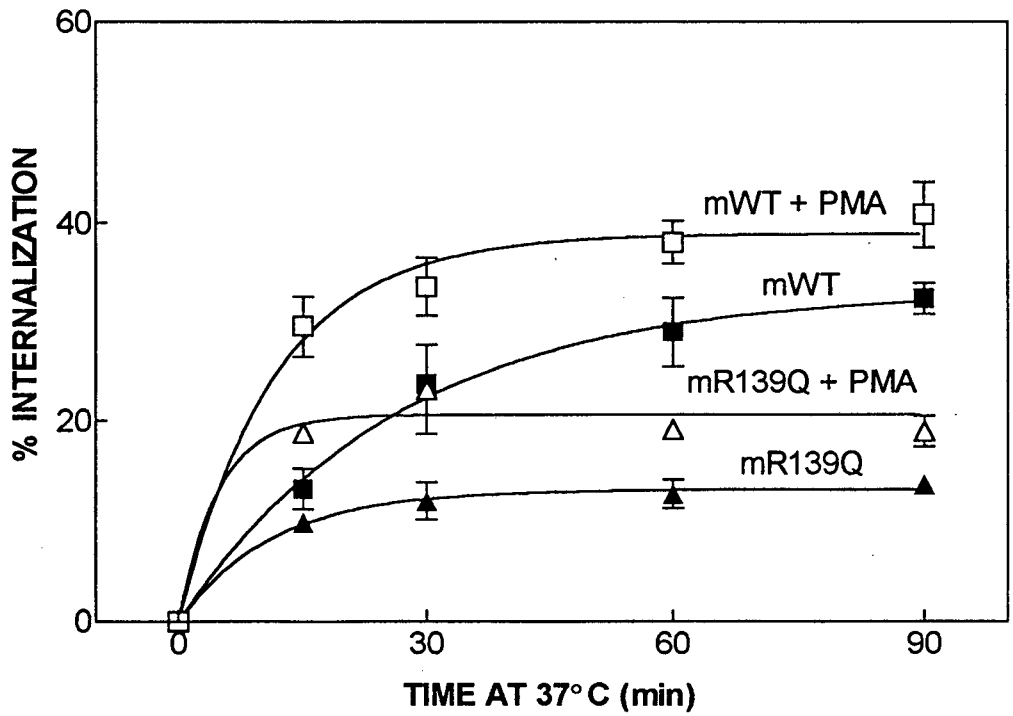


Figure 28. Internalization of ^{125}I-[D-Ala^6]GnRH mediated by WT, I143A, and I143L human GnRHRs

Internalization of ^{125}I-[D-Ala^6]GnRH in COS-1 cells expressing hWT (■), hI143A (O), and hI143L (Δ) GnRHRs was determined by the acid-wash method as described in **Section 2.7**. Internalized radioligand is expressed as a % of total cell-associated radioligand. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each time-point. Data points are the mean ± S.E.M. of three independent experiments performed in triplicate.

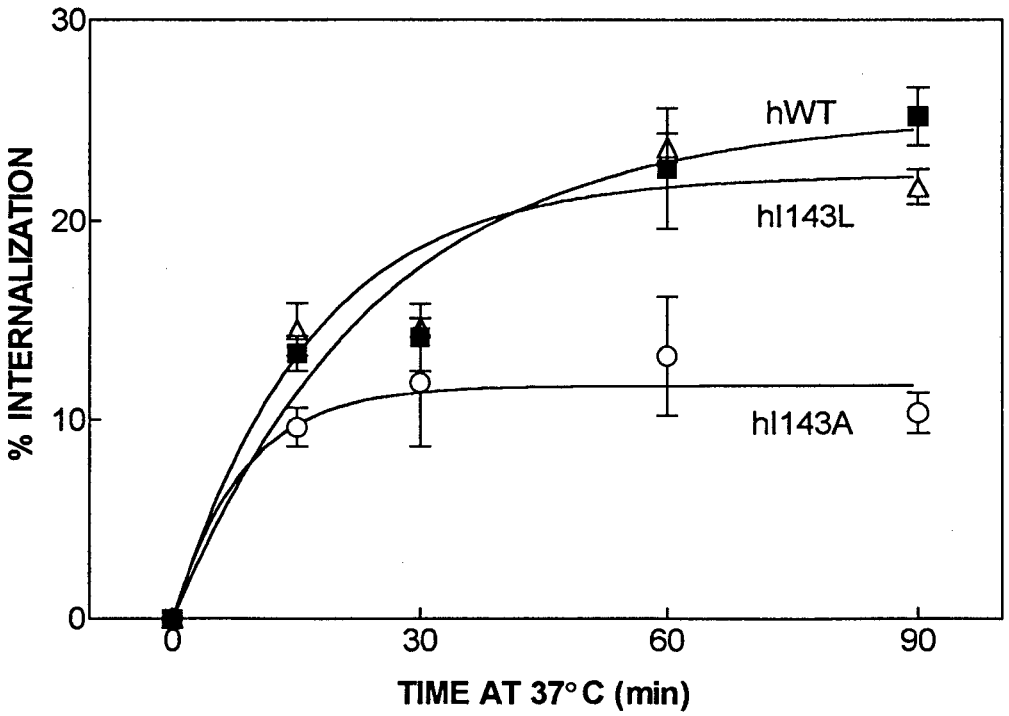


Figure 29. Models for G protein-coupled receptor activation

Schematic arrangement of the (a) *arginine switch* model of Oliveira et al., 1994, (b) model of Scheer et al., 1996, and (c) *arginine cage* model of Ballesteros et al., 1998. The seven helix bundle is viewed from the cytosolic side, and the helices are shown as cylinders. The angle of the helices are arbitrary. The shaded area represents the highly polar pocket formed between $Asn^{1.50}$, $Asp^{2.50}$, $Asp^{7.49}$, and $Tyr^{7.53}$ as described in text. The alternative positions of the arginine side-chain are connected by an arrow.

(a) Model of Oliveira et al., 1994

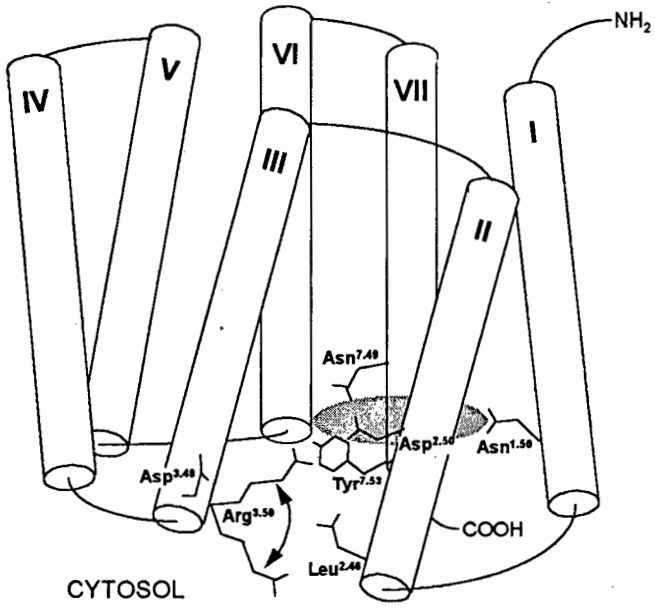
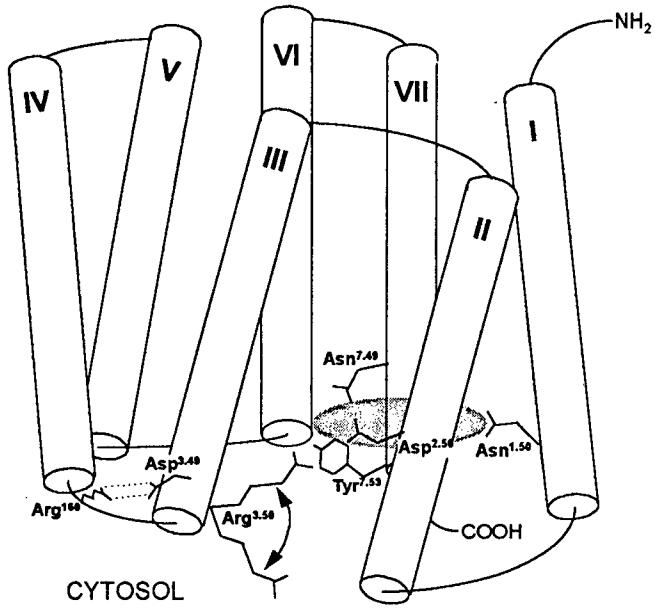


Figure 29. (continued). Models for G protein-coupled receptor activation

(b) Model of Scheer et al., 1996



(c) Model of Ballesteros et al., 1998

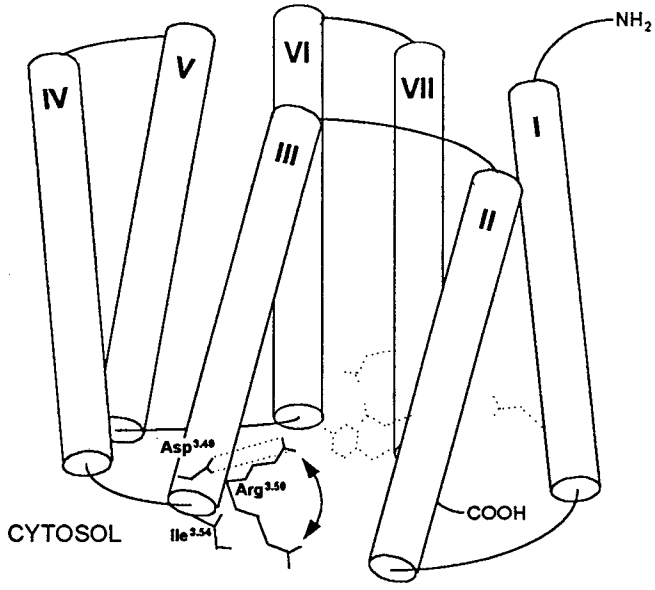


Figure 30. Sequence of the chicken GnRH receptor cytoplasmic tail mutants

The top line shows the sequence of the wild type chicken GnRH receptor C-terminal cytoplasmic tail. Sequence of the truncated mutants and of the point mutations are indicated below; the mutated amino acids are shown in bold-print.

	337	346	351	356	362	363	366	369	370
Wild-type:	SFREDVQLCLRGIEAAISQHVRHKPISVSEKTTKDGDVNGQVTSGGSNGTTVNTVC								
cS366Stop:	SFREDVQLCLRGIEAAISQHVRHKPISVSEKTTKDGDVNGQVTSGG								
cD356Stop:	SFREDVQLCLRGIEAAISQHVRHKPISVSEKTTKDG								
cT351Stop:	SFREDVQLCLRGIEAAISQHVRHKPISVSEK								
cS346Stop:	SFREDVQLCLRGIEAAISQHVRHKPI								
cS337Stop:	SFREDVQLCLRGIEAAI								
cS337A:	SFREDVQLCLRGIEAAI**A**QHVRHKPISVSEKTTKDGDVNGQVTSGGSNGTTVNTVC								
cT362S363AA:	SFREDVQLCLRGIEAAISQHVRHKPISVSEKTTKDGDVNGQV**AA**GGSNGTTVNTVC								
cS366A:	SFREDVQLCLRGIEAAISQHVRHKPISVSEKTTKDGDVNGQVTSGG**A**NGTTVNTVC								
cT369T370AA:	SFREDVQLCLRGIEAAISQHVRHKPISVSEKTTKDGDVNGQVTSGGSNG**AA**VNTVC								

Figure 31. Time-course of binding of ^{125}I-[His^5, D-Tyr^6]GnRH

Intact COS-1 cells expressing hWT (■), and cWT (○) GnRHRs were incubated with the agonist radioligand ^{125}I-[His^5, D-Tyr^6]GnRH (10^5 cpm/well) for 6 hrs at 0°C. At various time-points cells were washed, solubilized in 1 N NaOH and the bound radioactivity counted. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each time-point. The experiment shown is representative of two independent experiments performed in duplicate. The binding affinities (K_d) for [His^5, D-Tyr^6]GnRH for hWT and cWT receptors were, 5.07 nM and 3.28 nM respectively, while the calculated receptor numbers were 54.3±1.8 and 60.1±9.2 fmol/well respectively (K_d values and receptor numbers were determined by J. Lopes in our laboratory).

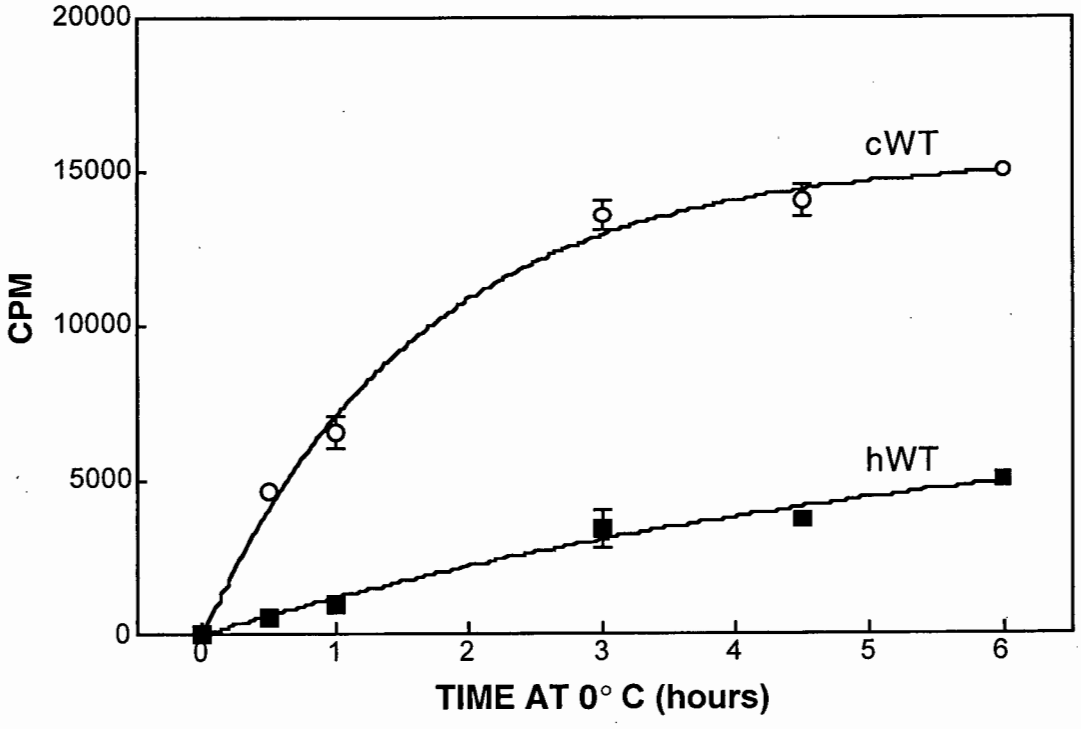
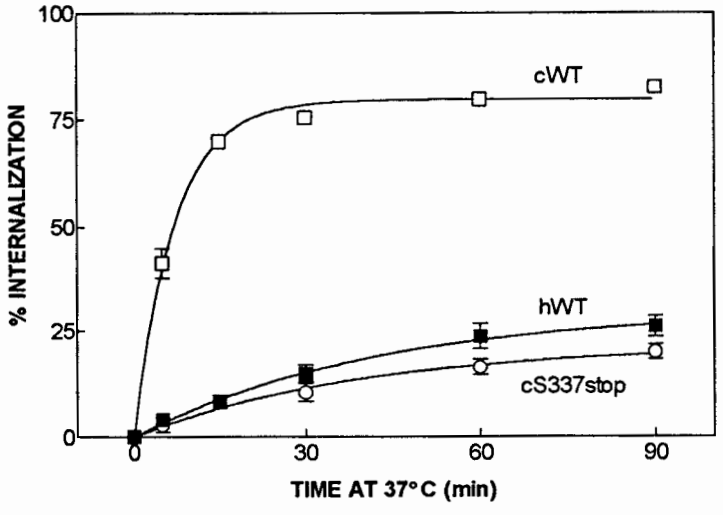


Figure 32. Comparison of internalization of WT human, WT chicken, and S337stop chicken GnRHRs

Internalization of ^{125}I-[His^5, D-Tyr^6]GnRH in COS-1 cells expressing hWT (■), cWT (□), and cS337stop (O) GnRHRs was determined by the acid-wash method as described in **Section 2.7**. Non-specific binding determined in parallel using untransfected COS-1 cells has subtracted at each time-point. Data points are the mean ± S.E.M. of four independent experiments performed in triplicate.

(a) Internalized radioligand expressed as a % of total cell-associated radioligand.



(b) Internalized radioligand (raw data)

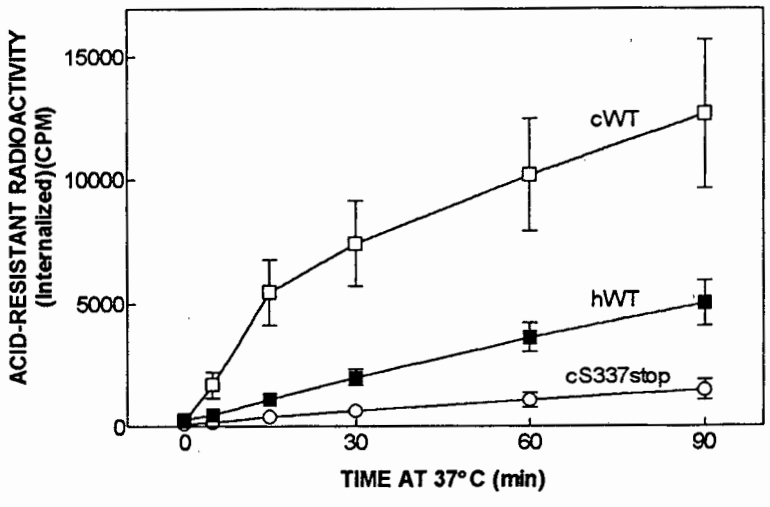


Figure 32. (continued) Comparison of internalization of WT human, WT chicken, and S337stop chicken GnRHRs

(c) Surface-bound radioligand

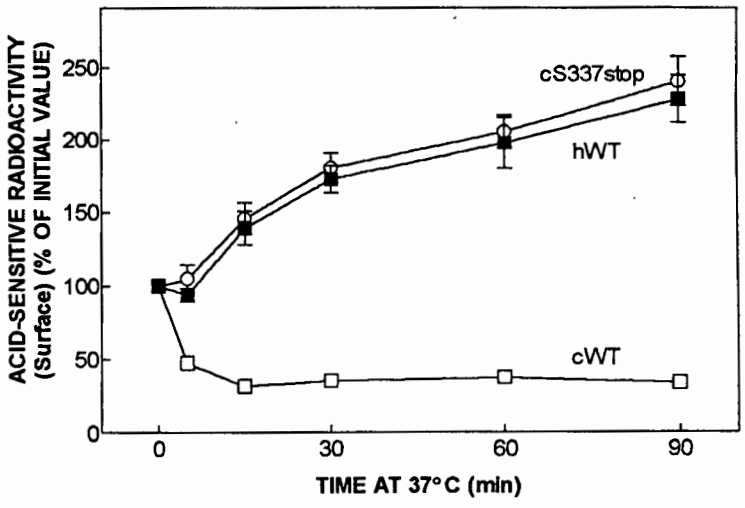


Figure 33. Internalization of ^{125}I-[His^5, D-Tyr^6]GnRH mediated by chicken GnRH receptors with truncations of the C-terminal tail

Internalization of ^{125}I-[His^5, D-Tyr^6]GnRH in COS-1 cells expressing cWT (■), cS337stop (O), cS346stop (Δ), cT351stop (∇), cD356stop (●), cS366stop (▲)GnRHRs was determined by the acid-wash method as described in **Section 2.7**. Internalized radioligand is expressed as a % of total cell-associated radioligand. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each time-point. Data points are the mean ± S.E.M. of two to three independent experiments performed in triplicate.

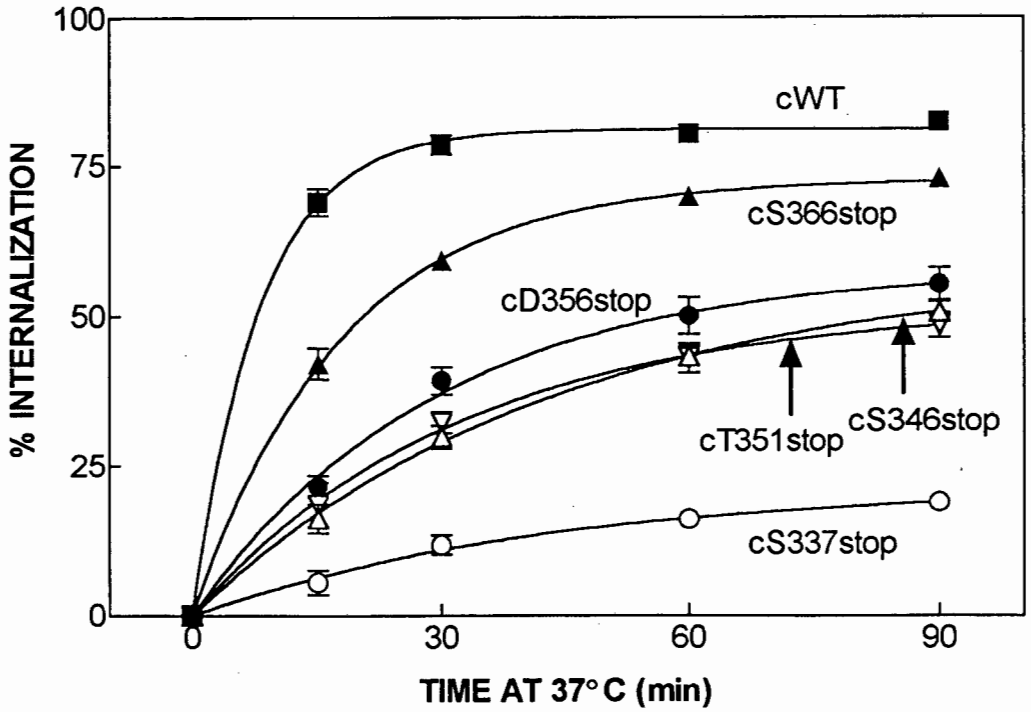


Figure 34. Effect of progressive truncations of cytoplasmic tail of chicken GnRH receptor on internalization and recycling rate constants

Internalization (■) and recycling (□) rate constants were calculated using equations 9 and 10 (see **Section 2.9.1**).

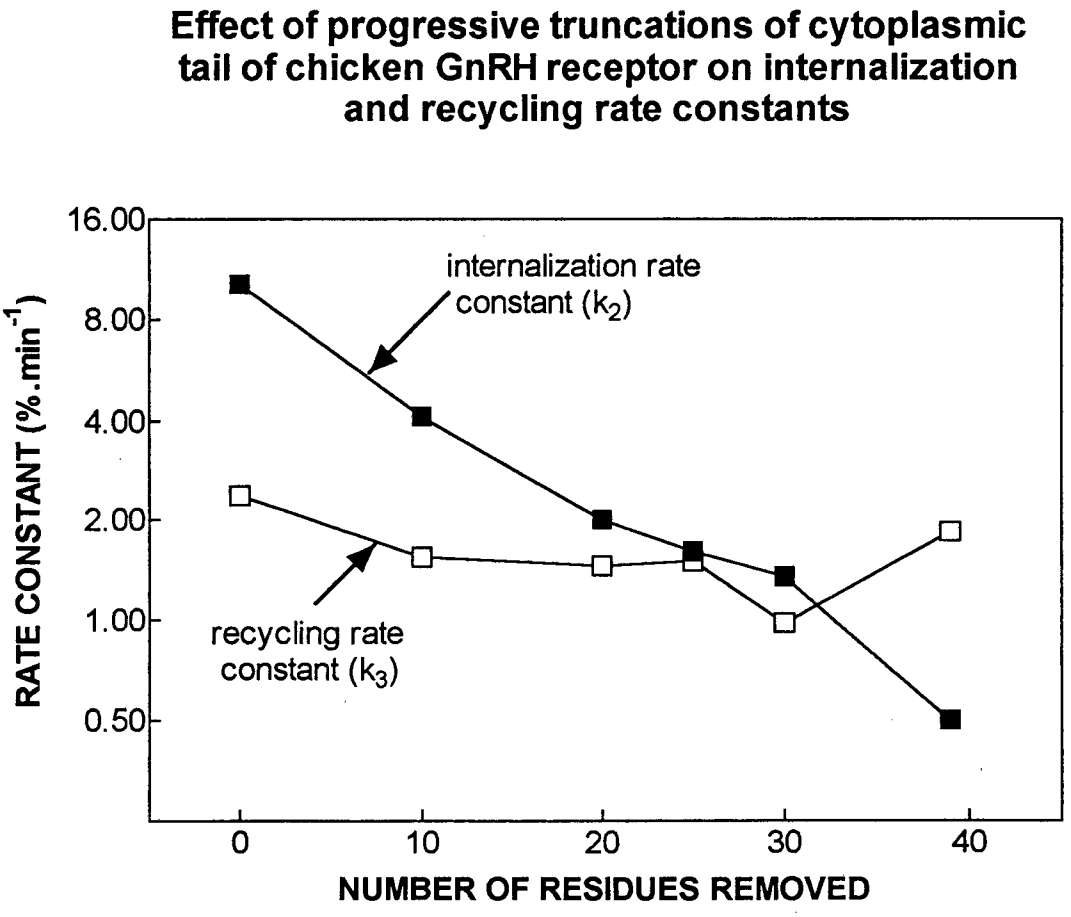


Figure 35. Internalization of ^{125}I-[His^5, D-Tyr^6]GnRH mediated by chicken GnRH receptors with point-mutations of Ser/Thr residues in the C-terminal tail

Internalization of ^{125}I-[His^5, D-Tyr^6]GnRH in COS-1 cells expressing cWT ( ), cS337A (O), cT362S363→AA (Δ), cS366A (∇), cT369T370→AA (●) GnRHRs was determined by the acid-wash method as described in **Section 2.7**. Internalized radioligand is expressed as a % of total cell-associated radioligand. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each time-point. Data points are the mean ± S.E.M. of two to three independent experiments performed in triplicate.

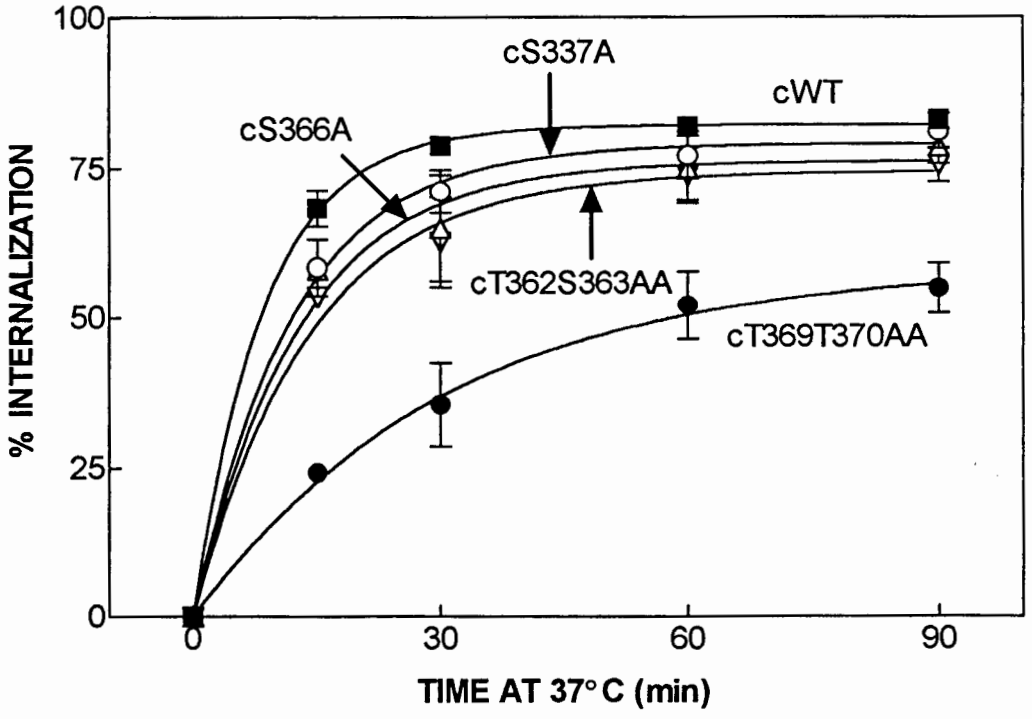


Figure 36. Internalization of ^{125}I-[His^5, D-Tyr^6]GnRH mediated by WT chicken, GnRHR in the presence of co-expressed WT dynamin I and mutant dynamin I-K44A

Internalization of ^{125}I-[His^5, D-Tyr^6]GnRH in COS-1 cells expressing cWT (■), cWT + dynamin I (0.5 μg DNA, ratio of 0.5 : 0.5 dynamin:cGnRHR) (○), and cWT + dominant-negative mutant dynamin I-K44A (0.5 μg DNA, ratio of 0.5 : 0.5 K44A:cGnRHR) (Δ) was determined by the acid-wash method as described in **Section 2.7**. Internalized radioligand is expressed as a % of total cell-associated radioligand. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted at each time-point. Data points are the mean ± S.E.M. of three independent experiments performed in triplicate.

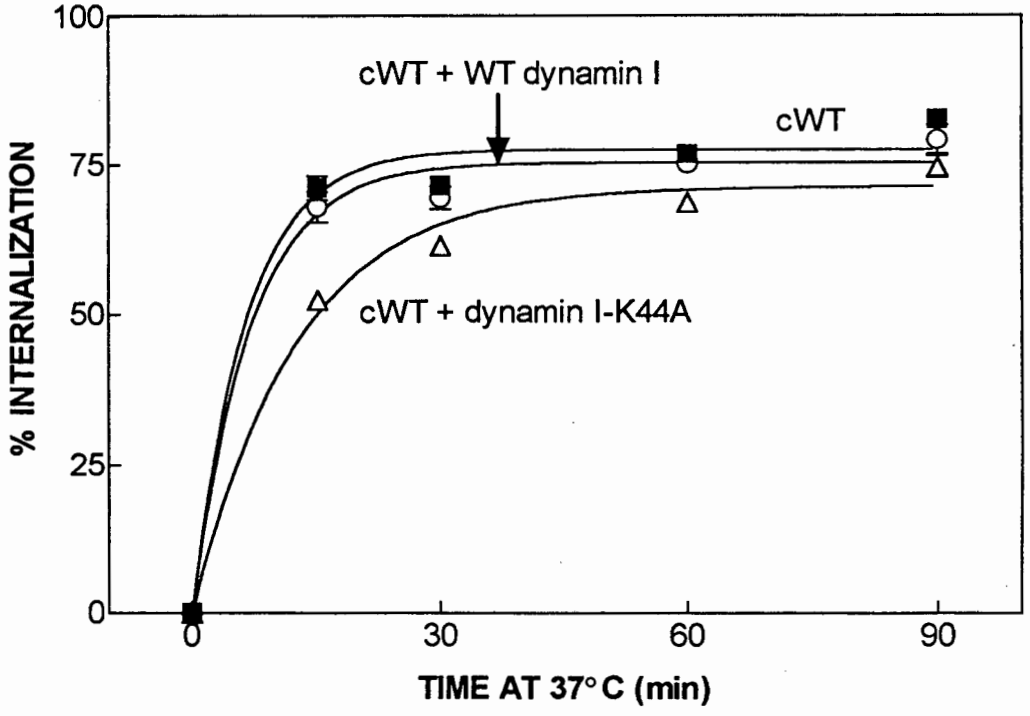


Figure 37. Internalization of ^{125}I-[His^5, D-Tyr^6]GnRH mediated by WT chicken, human, and truncated chicken GnRHRs in the presence of co-expressed dominant-negative mutant dynamin I-K44A

Internalization of ^{125}I-[His^5, D-Tyr^6]GnRH for 30 min in COS-1 cells expressing cWT (□), hWT (■), and cS337stop (stripped bars), in the presence of increasing amounts of co-transfected mutant dynamin I-K44A, was determined by the acid-wash method as described in **Section 2.7**. Internalized radioligand is expressed as a % of the internalization of the cWT receptor in the absence (0 μg) of co-transfected mutant dynamin I-K44A. Non-specific binding determined in parallel using untransfected COS-1 cells has been subtracted from the data. Data points are the mean ± S.E.M. of two independent experiments performed in duplicate.

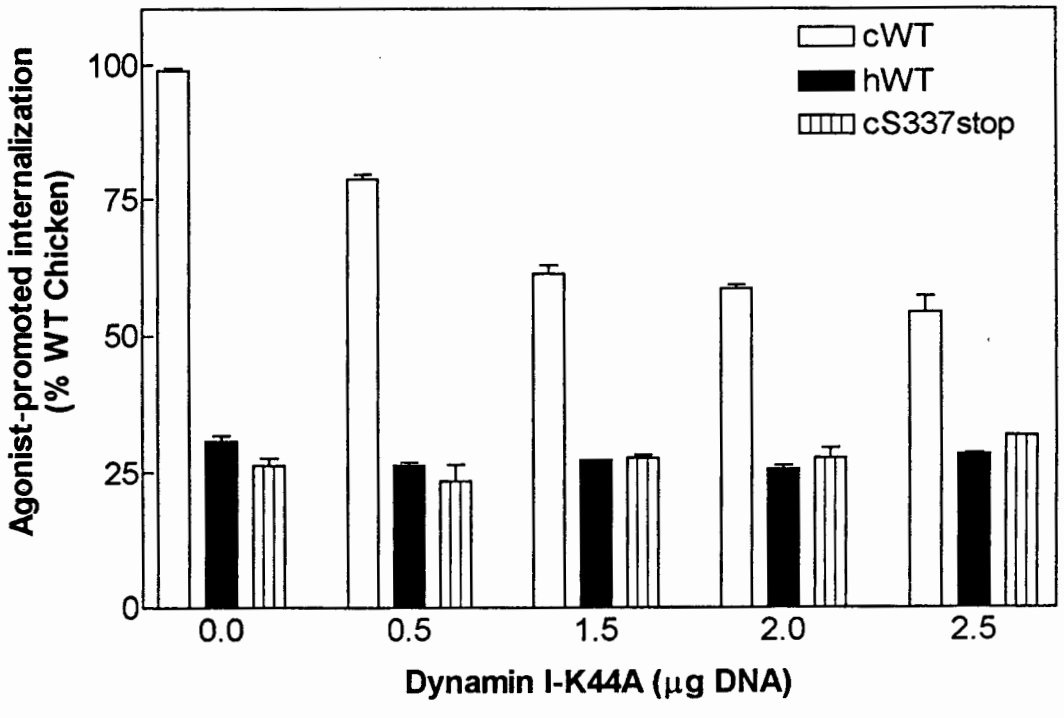


Figure 38 Theoretical receptor internalization curves

Curves were generated from the model described in **Section 2.9.1** in which receptor trafficking is treated in terms of an internalization rate constant, k_2 , and a recycling rate constant, k_3 . The values here were chosen to be similar to the chicken GnRH receptor.

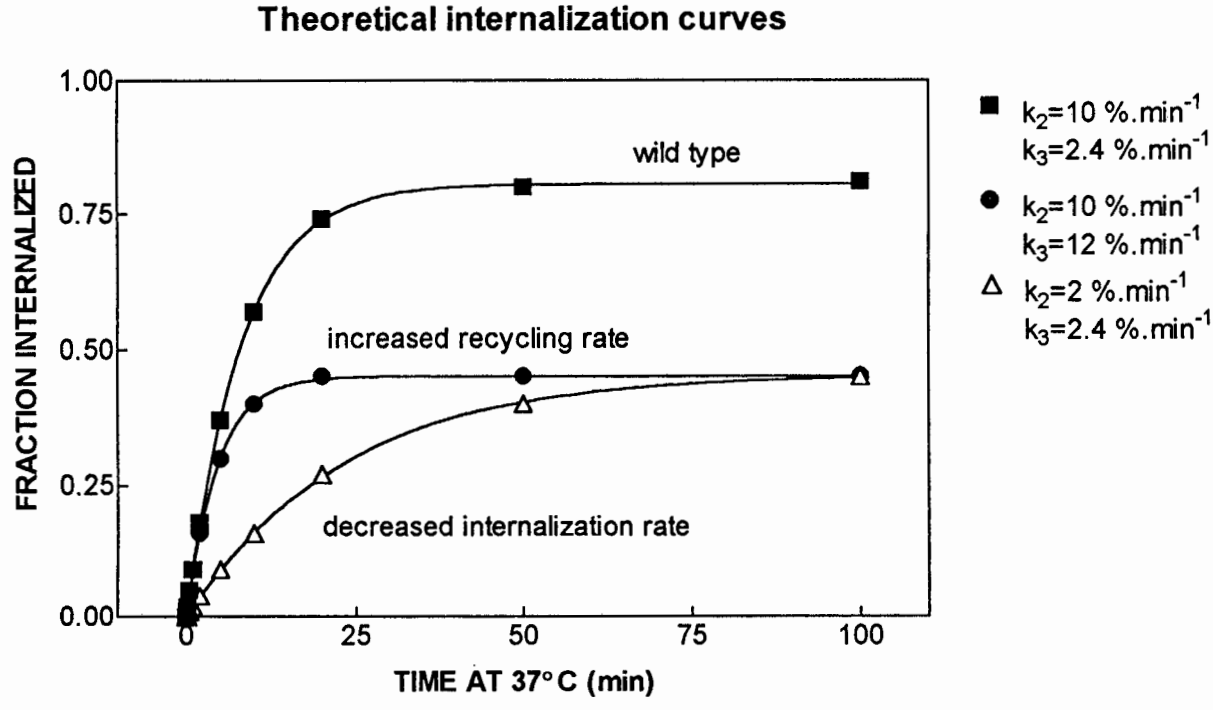


Figure 39. SDS-PAGE autoradiograph of photoaffinity labeled mWT GnRH receptors endogenously expressed on intact αT3-1 cells

Intact αT3-1 mouse pituitary cells were photoaffinity labeled (**Section 2.8.1**) and the membranes were solubilized in 1% Trition X-100 and stored at -70°C (lanes 1 and 2), or deglycosylated (37°C, 18 hrs) with peptide N-glycosidase F (PN-gase F, 8 units/ml), prior to being stored at -70°C (lanes 3 and 4). Pairs of lanes represent duplicate samples.

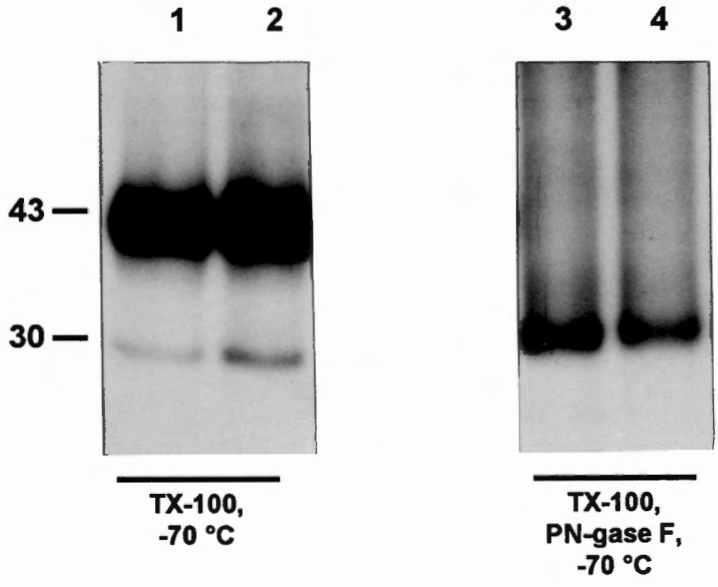


Figure 40. The effect of protease inhibitors on degradation of photoaffinity labeled GnRH receptors endogenously expressed on intact αT3-1 cells

Intact αT3-1 mouse pituitary cells were photoaffinity labeled (**Section 2.8.2**) and the labeled membranes were incubated at 37°C in the absence or presence of the protease inhibitors, leupeptin (0.1 mM) and PMSF (1 mM), for the indicated time-points, before solubilization, followed SDS-PAGE and autoradiography. Pairs of lanes represent duplicate samples.

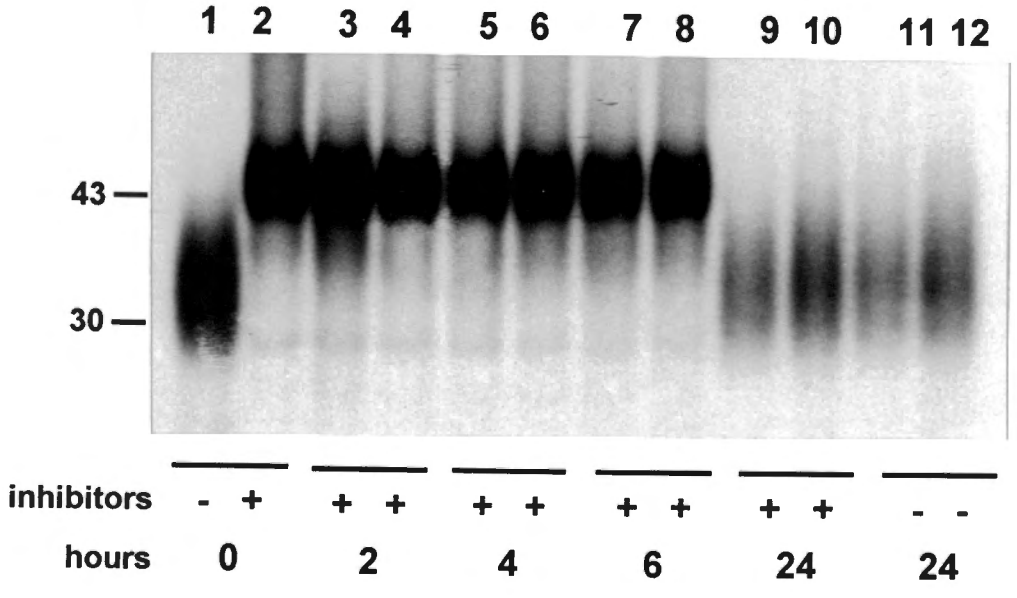


Figure 41. Stability of the covalent cross-link between photoaffinity ligand and GnRH receptor

The radioactivity in the GnRH receptor bands in lanes 2-10 of the gel presented in Figure 40 was quantitated and the data fitted by non-linear regression to a exponential decay equation, yielding a value of 0.05%.min^{-1} for the *in vitro* decay rate constant, corresponding to a half-life of 23.2 hrs. Data points are the mean ± S.E.M. of duplicate samples in a single experiment.

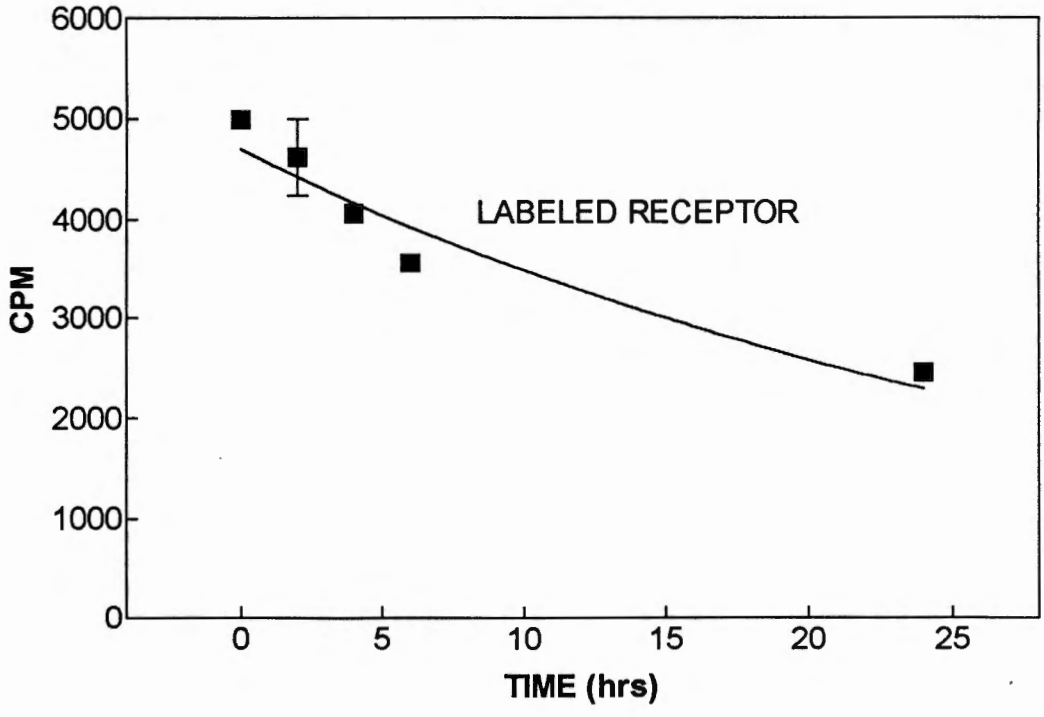


Figure 42. The effect of protease inhibitors on degradation of photoaffinity labeled GnRH receptors endogenously expressed on intact αT3-1 cells

Intact αT3-1 mouse pituitary cells were photoaffinity labeled (**Section 2.8.2**) and the labeled membranes incubated at 37°C in the absence or presence of the protease inhibitors, leupeptin (0.1 mM), PMSF (1 mM), and EDTA (1 mM), for the indicated time-points. Membranes were then solubilized and subjected to SDS-PAGE and autoradiography. Pairs of lanes represent duplicate samples.

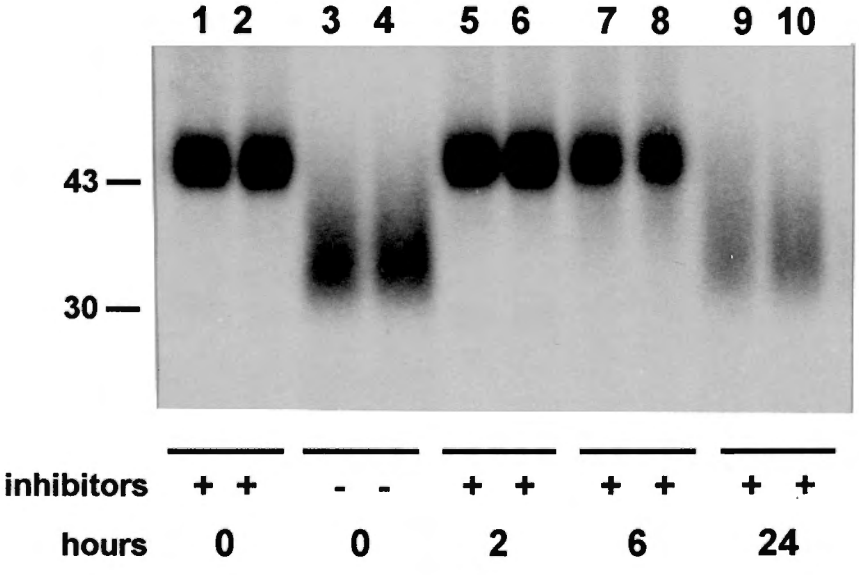


Figure 43. The effect of heat pre-treatment on degradation of photoaffinity labeled GnRH receptors endogenously expressed on intact αT3-1 cells

Intact αT3-1 mouse pituitary cells were photoaffinity labeled (**Section 2.8.2**) and the labeled membranes incubated at 37°C for the indicated time-points. In lanes 1-4 a 10 min heat inactivation step (60°C) was added prior to incubation at 37°C. Following incubation at 37°C, membranes were solubilized and subjected to SDS-PAGE and autoradiography. Pairs of lanes represent duplicate samples.

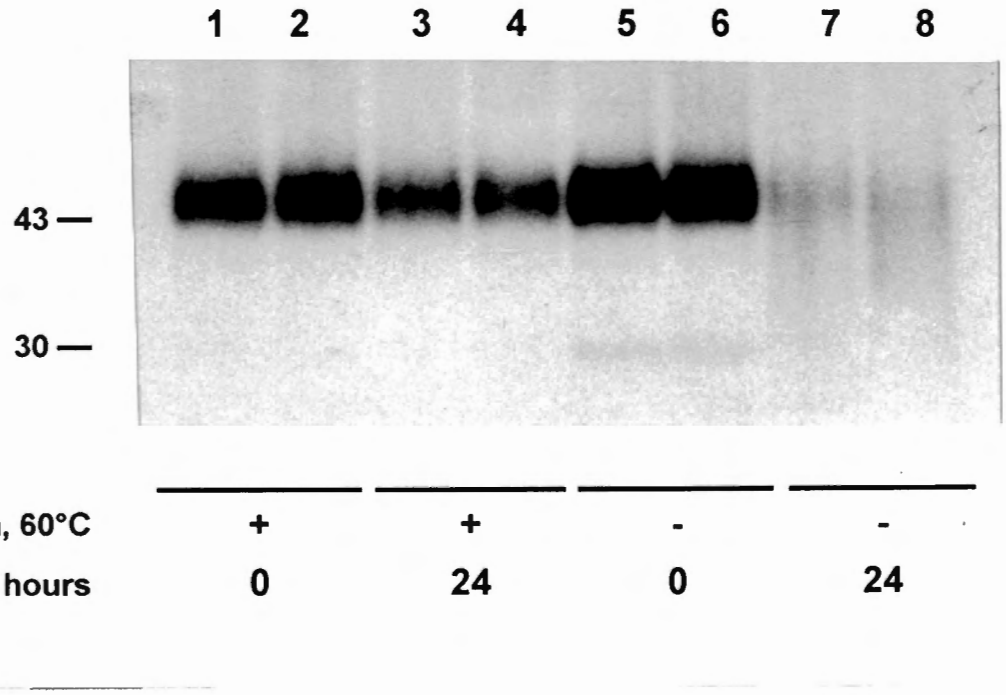


Figure 44. Intracellular degradation of ^{125}I-[*N*-azidobenzoyl-D-Lys^{6}]GnRH cross-linked receptor *invivo*

SDS-PAGE autoradiograph of photoaffinity labeled GnRH receptors endogenously expressed on intact αT3-1 cells. Following UV irradiation and cross-linking of ^{125}I-[*N*-azidobenzoyl-D-Lys^{6}]GnRH, cells were incubated at 37°C in HEPES/DMEM for the indicated times, after which they were processed as described in **Section 2.8.3**. Figure 45 shows quantitation of this data. Pairs of lanes represent duplicate samples.

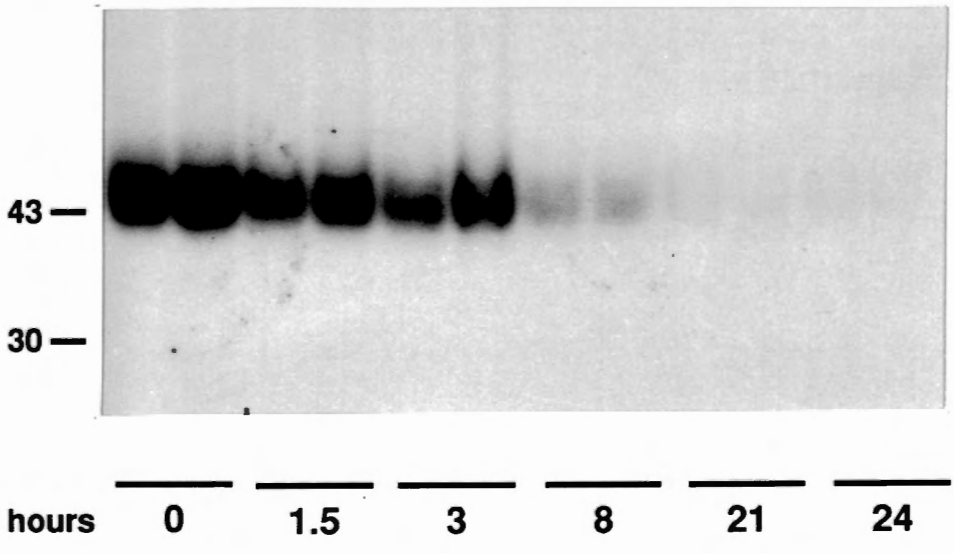


Figure 45. Intracellular degradation of ^{125}I-[*N*-azidobenzoyl-D-Lys^{6}]GnRH cross-linked receptor *in vivo*

^{125}I-[*N*-azidobenzoyl-D-Lys^{6}]GnRH radioactivity in gel bands as labeled GnRH receptor (■), radioactivity released into the medium (□), and total radioactivity at each time-point (○) is shown for the experiment in Figure 44. The experiment was performed as described in **Section 2.8.2**. Data points are the mean ± S.E.M. of duplicate dishes in a single experiment.